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Faculdade de Medicina

New Developments on the Pathogenesis of Systemic Lupus Erythematosus

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Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, especialidade Pediatria

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PREFACE

During my Pediatrics residency I had the invaluable opportunity to do a Pediatric Rheumatology internship at the Columbia University Medical Center in New York. There, my interest in research arose from taking care of patients with juvenile-onset systemic lupus erythematosus. I performed a study on major infections in this group of patients and I realized that new biomarkers were urgently needed to guide the judicious use of immunosuppressive drugs, as well as new treatment strategies with fewer side effects. I was certain that I wanted to participate in this quest and try to bring new light to the management of these patients.

In order to reach this goal, I needed to improve my scientific knowledge and become familiarized with the modern molecular biology and biocomputational techniques. I was fortunate to be a part of the *Program of Advanced Medical Education* from the Gulbenkian Foundation. This Program was specifically designed for physicians who wanted to increase their body of scientific knowledge and reasoning in order to answer clinically relevant questions.

I chose Dr. Sullivan's laboratory at the Children's Hospital of Philadelphia – University of Pennsylvania Perelman School of Medicine to develop my project. Dr. Sullivan is a preeminent physician-scientist, with unparalleled expertise in the epigenome of systemic lupus erythematosus. Her research focused on defining pathways related to disease perpetuation, in order to convert lupus to a curable disease rather than a chronic tractable one. I was incredibly fortunate to find an exceptional mentor and an outstanding nurturing work environment.

During my PhD, I decided to focus on two of the major complications of systemic lupus erythematosus: lupus nephritis and macrophage activation syndrome. The heterogeneous clinical outcome of patients with lupus nephritis always intrigued me and, therefore, I wanted to study the kidney microRNA signature in order to better

understand its pathophysiology. The results from this project established new urinary biomarkers that reflect cell proliferation in lupus nephritis and opened new avenues for targeted therapies that I hope will translate into an improvement of the control of the disease in the near future.

I was also intrigued by the enigmatic hemophagocytes and by the overwhelming inflammatory state that occurs in macrophage activation syndrome. Collaborating with Dr. Behrens, who developed a mouse model of secondary hemophagocytic syndrome, I participated in the study of the transcriptome of hemophagocytes. Our results contributed to the revolutionary conclusion that hemophagocytes may have a regulatory, anti-inflammatory function.

Finally, along with other colleagues from the laboratory, I worked on defining the quantitative and qualitative dysregulation of the transcriptome of systemic lupus erythematosus.

Overall, my Ph.D. experience was tremendously positive and rewarding. From the numerous tangible and intangible assets I collected over the years, I have specifically learned how to structure my scientific reasoning, how to design and develop scientific projects and had first-hand experience with multiple techniques that I, personally, executed and ultimately mastered. In addition, and more importantly, I had the unique opportunity to meet extraordinary and inspiring scientists with whom I learned so much and with whom, I am certain, I will find ways to collaborate in future projects. All in all, now that I returned to my home institution, my goal is quite simple: better the care I provide to my patients by linking and synergizing my activities as a physician and as a scientist.

INTRODUCTORY NOTE

This thesis focuses on systemic lupus erythematosus and tries to bring different scientific approaches to the study of chronic inflammation.

First, a brief overview of the clinical characteristics of the disease is presented, followed by a chapter devoted to the pathophysiology of lupus. I am a co-author of a paper on the same topic published in *Nature Reviews Rheumatology*.

Chapter 3 is dedicated to the genetics and epigenetics of lupus and it integrates a paper published in *Current Rheumatology Reports*.

The global goals of this thesis are enumerated in chapter 4 and the subsequent chapters are the result of original work performed during the PhD:

- The results from the clinical project on major infections in juvenile-onset systemic lupus erythematosus are presented in Chapter 5. This study was published in the journal *Clinical Immunology.*

- The analysis of the kidney miRNA signature of lupus nephritis, which prompted the identification of a new pathway responsible for cell control, is addressed in Chapters 6 and 7. The results of this project were published in *Arthritis and Rheumatology*.

- Chapter 8 is devoted to the study of hemophagocytes, cells that are present in the activation macrophage syndrome, one of the most serious manifestations of SLE. The content of this chapter was published in another paper in *Arthritis and Rheumatology*.

- Finally, the project devoted to the study of the transcriptome of systemic lupus erythematosus is presented. These results were published in *PLOS ONE*.

All the papers are included as Appendices.

INDEX

ABBREVIATIONS

°C	Degrees Celsius
µL	Microliter
AAALAC	American Association for Accreditation of Laboratory Animal Care
ACL	Anticoagulant lupus antibody
ACP5	Acid phosphatase 5, tartrate resistant
ACR	American College of Rheumatology
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
AKT	V-Akt murine thymoma viral oncogene
ALPS	Autoimmune lymphoproliferative syndrome
AMP	Adenosine monophosphate
APRIL	A proliferation-inducing ligand
ARA	American Rheumatism Association
ATAC-seq	Assay for transposase-accessible chromatin sequencing
ATG5	Autophagy related 5
BAFF	B cell-activating factor
BANK1	B-cell scaffold protein with ankyrin repeats gene
BCR	B cell receptor
BILAG	British Isles Lupus Assessment Group
BIM	Bcl-2 interacting mediator of cell death
BLK	B lymphoid tyrosine kinase
BLyS	B lymphocyte stimulator
BRD4	Bromodomain containing 4
BUN	Blood urea nitrogen
B reg	B regulatory
CaMKIV	Calcium/calmodulin kinase IV
CCNE2	Cyclin E2
CCP	Cyclic citrullinated peptides
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
C. elegans	*Caenorhabditis elegans*
cGAMP	Cyclic GMP-AMP
CHFR	Complement factor H regulator
ChIP-Seq	Chromatin immunoprecipitation sequencing
CI	Confidence interval
CMV	Cytomegalovirus
COX6C	Cytochrome C oxidase subunit 6C
COX6A1	Cytochrome C oxidase subunit 6A1
CREMα	Cyclic adenosine monophosphate responsive element modulator
CT	Computorized tomography
CR	Complement receptor
CSK	C-src kinase
CTCF	Corrected total cell fluorescence
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCR5	C-X-C chemokine receptor type 5
DAI	DNA-dependent activator of IFN-regulatory factor 1
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dsDNA	Double stranded deoxyribonucleic acid
DZNep	3-deazaneplanocin A
E2F8	E2F transcription factor 8
ECLAM	The European Consensus Lupus Activity Measurement
EGF	Epidermal growth factor

ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal, regulated kinase
ESR	Erythrocyte sedimentation rate
ETS1	ETS proto-oncogene 1, transcription factor
EZH2	Enhancer of zeste homolog 2
FAS	Fas cell surface death receptor
FC	Fragment crystallizable
FSTL1	Follistatin-related protein 1
FTL	Ferritin, light polypeptide
g	Gram
GADD45α	Growth arrest and DNA damage inducible alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTP	Guanosine triphosphate
GWAS	Genome wide association studies
GX2	Genetic-screened homeobox 2
h	Hour
HAS2	Hyaluronic acid synthase 2
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIPAA	Health Insurance Portability and Accountability Act
HIVEP	Human immunodeficiency virus type I enhancer binding protein 1
HLA	Human leukocyte antigen
HMGB1	High-mobility group box protein 1
HNF4A	Hepatocyte nuclear factor 4 alpha
ID4	Inhibitor of DNA binding 4
Ig	Immunoglobulin
IKZF1	IKAROS family zinc finger 1
IPA	Ingenuity Pathway Analysis
INF	Interferon
INFAR	Interferon α/β receptor
iNOS	Inducible nitric oxid synthase
IRAK1	Interleukin 1 receptor associated kinase 1
IRF1	Interferon regulatory factor 1
HIV	Human immunodeficiency virus
ICAM1	Intercellular adhesion molecule 1
IFITM2	Interferon induced transmembrane protein 2
IL	Interleukin
INR	International normalized ratio
IPS1	Interferon-β stimulator 1
IRAK1	Interleukin-1 receptor associated kinase 1
IRB	Institutional review board
IRF	Interferon regulator factor
ITGAM	Integrin subunit alpha M
IV	Intravenous
IVNS1ABP	Influenza virus NS1A binding protein
JAK	Janus kinases
jSLE	Juvenile-onset systemic lupus erythematosus
KD	Knock-down
KEGG	Kyoto Encyclopedia of Genes and Genomes
KLF13	Kruppel-Like factor 13
KO	Knock-out
L1	Long interspersed nuclear element 1
LAMP1	Lysosomal-associated membrane protein 1
LDH	Lactate dehydrogenase
lncRNA	Long non coding RNA
LPS	Lipopolysaccharides
LR	Likelihood ratio

LYN	Tyrosine protein kinase Lyn
LYST	Lysosomal trafficking regulator
MAD2L1	Mitotic arrest deficient 2-like protein 1
MCP1	Monocyte chemoattractant protein 1
MECP2	Methyl-CpG binding protein 2
Mda-5	Melanoma differentiation-associated protein 5
Mg	Milligram
MHC	Major histocompatibility complex
miRNA	microRNA
mL	Milliliter
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRI	Magnetic resonance imaging
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYBL1	V-Myb avian myeloblastosis viral oncogene homolog-like 1
MyD88	Myeloid differentiation primary response gene 88
NA	Data not available
NADPH	Nicotinamide adenine dinucleotide phosphate
NCF2	Neutrophil cytosolic factor 2
NETs	Neutrophil extracellular traps
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL	Urinary neutrophil gelatinase-associated lipocalin
NIH	National Institute of Health
NK	Natural killer cell
NLRC	NLR family, CARD domain containing
NMDA	N-methyl D-aspartate
NSAID	Nonsteroidal anti-inflammatory drug
OR	Odds ratio
PAD4	Peptylarginine deiminase
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor receptor
PD-L1	Programmed death ligand 1
PET	Positron emission tomography
PI	Propidium iodide
POLQ	DNA polymerase theta
PRKCB	Protein kinase C, Beta
PTEN	Phosphatase and tensin homolog
PTPN22	Protein phosphatase nonreceptor type 22
qRT-PCR	quantitative real-time polymerase chain reaction
RAB27A	RAB27A, member RAS oncogene family
RACGAP1	Rac GTPase activating protein 1
RANTES	Regulated on activation, normal T cell expressed and secreted
RasGRP1	Ras guanylnucleotide releasing protein 1
RFX1	Regulatory factor X1
RIG1	Retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
RMA	Robust multi-array
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SAA3	Serum amyloid 3
SELENA-SLEDAI	The Safety of Estrogens in Lupus Erythematosus: National Assessment-SLEDAI
SH2D1A	SH2 domain containing 1A
sJIA	Systemic juvenile idiopathic arthritis
SLAM	Systemic Lupus Activity Measure

SLC5A11	Solute carrier family 5 member 11
SLC15A4	Solute carrier family 15 member 4
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLICC	Systemic Lupus International Collaborating Clinics
SNAPC3	Small nuclear RNA activating complex polypeptide 3
SNP	Single nucleotide polymorphisms
snRNP	Small nuclear ribonucleoproteins
SNTB1	Syntrophin beta 1
ST2	Suppression of tumorigenicity 2
STAT4	Signal transducer and activator of transcription 4
STING	Stimulator of interferon genes protein
STX11	Syntaxin 11
STXBP2	Syntaxin binding protein 2
SSA	Sjögren's-syndrome-related antigen A
SSB	Sjögren's-syndrome-related antigen B
SUMO	Small Ubiquitin-like Modifier
TBK1	Serine/threonine-protein kinase TBK1
TCR	T cell receptor
TET	Ten-eleven translocation
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
THBS1	Thrombospondin 1
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLK2	Tousled Like Kinase 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNIP1	TNFAIP3 interacting protein 1
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TNFSF4	Tumor necrosis factor (Ligand) superfamily, member 4
TRAM	Toll-interleukin 1 receptor domain containing adaptor protein
Treg	T regulatory
TREML4	Triggering receptor expressed on myeloid cells like 4
TRIF	TRIF-related adaptor molecule
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
TYK2	Tyrosine kinase 2
UBC	Ubiquitin C
UBE2L3	Ubiquitin conjugating enzyme E2 L3
UV	Ultraviolet
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XIAP	X-Linked inhibitor of apoptosis
Yrs	Years
ZAP-70	Tyrosine kinase z-associated protein 70

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a challenging autoimmune disease, with a complex etiopathogenesis and an unpredictable clinical course. In a large cohort of juvenile-onset SLE patients, we found that major infections were common, were associated with active disease and its treatment, and resulted in noteworthy morbidity. New biomarkers to guide the judicious use of immunosuppressive drugs and new treatment strategies with fewer side effects would, therefore, have an enormous impact in the management of these patients. In order to reach these goals we used the modern tools of molecular biology and focused on two of the most important complications of SLE: lupus nephritis and macrophage activation syndrome.

Firstly, we identified the kidney lupus nephritis specific microRNA (miRNA) signature, which reflected mainly cell proliferation. MiRNAs are noncoding RNAs responsible for post-transcriptional gene silencing. These key regulatory molecules control the expression of multiple genes, so its dysregulation can contribute to sustained pathology. We showed that miR-26a and miR-30b were significantly decreased in the kidneys and urine of lupus nephritis patients. *In vitro*, the knockdown of miR-26a and miR-30b caused the proliferation of human mesangial cells and increased the expression of genes related to the cell cycle, including *CCNE2, E2F8, MAD2L1, MYBL1* and *POLQ*.

The Human Epidermal Growth Factor Receptor 2 (HER2) is a protein previously known to regulate miR-26a and miR-30b expression. Trastuzumab, a monoclonal antibody against HER2, used in breast cancer treatment, produces therapeutic actions precisely by up-regulating miR-26a and miR-30b. In human mesangial cells we also found that trastuzumab increases these two miRNAs. We hypothesized that HER2 also played a role in the pathogenesis of lupus nephritis and indeed we identified a dramatic overexpression of HER2 in the glomeruli and tubular compartments of the kidneys of lupus nephritis patients. The same pattern was not seen in the kidneys of healthy individuals or in other proliferative glomerulonephritides, including post-streptococcus glomerulonephritis, IgA nephropathy and granulomatosis with polyangiitis. Furthermore, in the lupus-prone NZM2410 mice we identified a highly increased expression of HER2, which correlated with disease activity. Finally, we showed that urinary HER2 was

significantly increased in lupus nephritis and that its levels increased during flares, were higher in class III and class IV lupus nephritis and correlated with urinary protein-creatinine and monocyte chemoattractant protein 1 (MCP1) and vascular cell adhesion protein 1 (VCAM1) levels. We, therefore, established a strong rationale to use trastuzumab to block HER2 and decrease cell proliferation and damage in lupus nephritis.

Regarding the macrophage activation syndrome, we were interested in the characterization of hemophagocytes. These are activated macrophages that have engulfed other hematopoietic cells. Traditionally they have been associated with the development of cytopenias in several life-threatening cytokine storm syndromes. New data have challenged this concept, since pancytopenia occurs in the absence of hemophagocytosis in mice and, in humans, over 40% of patients with macrophage activation syndrome do not have hemophagocytes in bone marrow aspirates. On the other hand, subclinical hemophagocytosis is detected in more than 50% of patients with systemic juvenile idiopathic arthritis, but only 10% develop a life-threatening macrophage activation syndrome. Thus the function and significance of hemophagocytes remained mysterious.

Recent evidence demonstrated that environmental factors, particularly the cytokine milieu, determine the macrophage activation status in a continuum ranging from M1 to M2. M1 macrophages, driven by interferon γ, typically acquire pro-inflammatory properties and are associated with tissue damage, whereas M2 macrophages have more heterogeneous stimuli and functions, being associated with immunoregulation, tissue remodeling and fibrosis. Since interferon γ is the hallmark cytokine of hemophagocytic lymphohistiocytosis, one would expect to find that hemophagocytes express M1 surface receptors.

We characterized the transcriptional phenotype of mouse Toll-like receptor 9 (TLR9) – induced hemophagocytes and described the surface phenotype of human bone marrow hemophagocytes. Interestingly, murine hemophagocytes had up-regulation of genes associated with the M2 and not the M1 phenotype. Immunohistochemical analyses in bone marrow samples from a uniquely diverse cohort of patients with hemophagocytic syndromes showed universal staining of hemophagocytes for the M2 marker CD163, but rarely for CD206 or CD64. Collectively, these data support the

hypothesis that hemophagocytes have immunoregulatory functions and open new doors for the study of the pathogenesis of this syndrome.

Finally, we were interested in characterizing the transcriptome of SLE, by RNA-sequencing, in order to better understand the mechanisms that are responsible for chronicity. Monocytes from SLE patients exhibited a globally dysregulated gene expression. The transcriptome was not simply altered by the activation of a set of genes, but was also qualitatively different. Splicing patterns and polyadenylation were significantly altered and SLE monocytes expressed novel transcripts, an effect that was replicated by exposing control monocytes to lipopolysaccharide (LPS). We further identified increased circulating endotoxin in SLE patients, suggesting that chronic microbial translocation could contribute to the immunologic dysregulation in SLE, a new potential disease mechanism.

In conclusion, with these different projects, which globally focused on the transcriptome and epigenome of SLE, we identified novel pathways and challenged the current paradigms. We described new mechanisms of disease, including the role of LPS in transforming qualitatively the SLE transcriptome and a putative immunoregulatory function for hemophagocytes. We demonstrated that miR-26a, miR-30b and HER2 control cell proliferation in lupus nephritis and that are promising biomarkers. Most importantly, our work rose the possibility of using anti-HER2 drugs for lupus nephritis management, opening the door to a new treatment strategy in this disease.

Keywords: Systemic lupus erythematosus; lupus nephritis; miR-26a; miR-30b; HER2; trastuzumab; macrophage activation syndrome; hemophagocytes.

RESUMO

O lúpus eritematoso sistémico é uma doença autoimune com uma patogénese complexa e uma apresentação clínica muito variável. Numa coorte de crianças e adolescentes com lúpus eritematoso sistémico demonstrámos que as infecções major são comuns, associam-se à atividade de doença e ao seu tratamento e levam a importante morbilidade. É, assim, essencial identificar novos biomarcadores para guiar a utilização de fármacos imunossupressores, e desenvolver novas estratégias terapêuticas com menos efeitos adversos. Explorámos as potencialidades da biologia molecular para tentar atingir estes objectivos, focando-nos em duas das mais graves complicações do lúpus eritematoso sistémico: a nefrite lúpica e a síndrome de ativação macrofágica.

Em primeiro lugar, descrevemos o padrão renal de microRNAs (miRNAs) característico da nefrite lúpica. Os miRNAs controlam a expressão de múltiplos genes, impedindo a tradução dos mRNAs aos quais se ligam. Os miRNAs são, assim, moléculas reguladoras essenciais, cuja desregulação pode levar à doença e contribuir para a sua cronicidade. Demonstrámos existir na nefrite lúpica uma profunda desregulação do habitual padrão de miRNAs, o que certamente contribuirá para a patogénese da doença. Globalmente, este padrão de miRNAs associa-se a proliferação celular. Identificámos, em particular, uma diminuição significativa do miR-26a e do miR-30b nos rins e na urina de doentes com nefrite lúpica. Demonstrámos em células mesangiais humanas que a diminuição destes miRNAs causa um aumento da expressão de genes relacionados com o ciclo celular, incluindo *CCNE2, E2F8, MAD2L1, MYBL1* e *POLQ,* e, consequentemente, proliferação celular.

O receptor do factor de crescimento epidérmico humano 2 (HER2) regula a expressão do miR-26a e do miR-30b. O trastuzumab, um anticorpo monoclonal contra o HER2, é usado no tratamento do cancro da mama, sendo a sua ação eficaz justamente porque aumenta os níveis de miR-26a e de miR-30b. Em células mesangiais humanas constatámos que o trastuzumab também aumenta a expressão deste dois miRNAs. Levantámos, assim, a hipótese de que a proteína HER2 participaria na patogénese da nefrite lúpica. Efetivamente encontrámos um aumento muito significativo da expressão

da proteína HER2 nos glomérulos e nos túbulos de doentes com nefrite lúpica. O mesmo padrão não foi detetado em rins de pessoas saudáveis e em doentes com glomerulonefrite pós-estreptocócica, nefropatia IgA e granulomatose com poliangeíte. Acresce que em ratinhos NZM2410 também encontrámos um aumento franco da expressão da proteína HER2, correlacionando-se esta com a atividade da doença. Finalmente, demonstrámos que a proteína HER2 se encontra aumentada na urina de doentes com nefrite lúpica e que os seus níveis aumentam em períodos de surto da doença, sendo mais elevados na nefrite lúpica classe III e classe IV e correlacionando-se com a proteinúria/creatinúria, o MCP1 e o VCAM1. Este estudo demonstrou claramente que a proteína HER2 participa no controlo da proliferação celular e, por conseguinte, da lesão renal na nefrite lúpica. Estabeleceu-se, assim, a base científica para o estudo do trastuzumab na terapêutica da nefrite lúpica.

Relativamente à síndrome de ativação macrofágica, o objectivo primordial era a caracterização dos hemofagócitos. Estes são macrófagos ativados que fagocitaram outras células hematopoiéticas. Classicamente os hemofagócitos têm sido associados à ocorrência de citopénias em situações de libertação catastrófica de citocinas. Contudo, novos dados têm vindo a pôr em causa este conceito, uma vez que a pancitopénia ocorre na ausência de hemofagocitose e hemofagocitose subclínica ocorre em mais de 50% dos doentes com artrite idiopática juvenil sistémica e apenas 10% desenvolve clinicamente a síndrome de ativação macrofágica. A função dos hemofagócitos permanece, assim, por determinar.

Fatores ambientais, em particular o padrão de citocinas, determinam o grau de ativação macrofágica num *continuum* de M1 até M2. Os macrofágos M1 têm, tipicamente, propriedades pró-inflamatórias e associam-se a lesão dos tecidos. Os macrófagos M2 têm funções mais variadas, incluindo a imunoregulação, a remodelação dos tecidos e a fibrose.

O interferão γ estimula os macrofágos a adotarem um fenótipo M1. Tendo em consideração que esta citocina se encontra elevada nas síndromes hemofagocíticas, será expectável que os hemofagócitos se assemelhem a macrófagos M1 e expressem os seus recetores de superfície, tendo, assim, um fenótipo pró-inflamatório.

Caracterizámos o transcriptoma de hemofagócitos de ratinho induzidos pela estimulação repetida de TLR9. Surpreendentemente, estes tinham um aumento da

expressão de genes associados ao fenótipo M2 e não M1. Por imunohistoquímica estudámos os receptores de superfície de hemofagócitos de uma coorte diversificada de doentes com síndromes hemofagocíticas. Demonstrámos que os hemofagócitos expressavam universalmente CD163, um marcador M2, e, em raras ocasiões, expressavam CD206 e CD64. Globalmente estes dados apoiam a hipótese de que os hemofagócitos têm funções imunoreguladoras e abrem a porta à realização de novos estudos sobre a função destas células.

Finalmente, pretendíamos caracterizar o transcriptoma do lúpus eritematoso sistémico, usando a técnica de sequenciação de RNA, com o intuito último de tentar identificar mecanismos responsáveis pela cronicidade da doença. Constatámos que monócitos de doentes com lúpus eritematoso sistémico apresentavam uma desregulação global da expressão genética. O transcriptoma não só estava alterado quantitativamente, por ativação e repressão de diferentes genes, mas também qualitativamente. Detetaram--se novos padrões de processamento de RNA e novas formas de RNA, que foram replicadas pela exposição de monócitos de controlo ao lipopolissacarídeo. Finalmente, identificámos um aumento em circulação do lipopolissacarídeo em doentes com lúpus eritematoso sistémico. A translocação microbiana crónica pode, assim, contribuir para a desregulação imunológica própria desta doença.

Em conclusão, com estes diferentes projetos, que globalmente se focaram no transcriptoma e no epigenoma do lúpus eritematoso sistémico, identificámos novas vias e desafiámos alguns dos paradigmas vigentes. Descrevemos novos mecanismos de doença, incluindo o papel do lipopolissacarídeo na transformação qualitativa do transcriptoma no lúpus eritematoso sistémico e a possibilidade dos hemofagócitos terem funções imunoreguladoras. Demonstrámos ainda que miR-26a, miR-30b e HER2 controlam a proliferação celular na nefrite lúpica e são promissores biomarcadores. Mais importante ainda o nosso trabalho levantou a hipótese de se usarem fármacos anti-HER2 para o tratamento da nefrite lúpica, uma nova estratégia terapêutica para o controlo desta doença.

CHAPTER 1

Clinical Review of Systemic Lupus Erythematosus

1.1 OVERVIEW

Systemic Lupus Erythematosus (SLE or lupus) is a chronic autoimmune disease with a highly diverse clinical course, characterized by periods of relapses and remissions. Patients present with a wide array of symptoms, signs, and laboratory findings and have a variable prognosis that depends upon the type and severity of organ involvement. The most common clinical manifestations are constitutional complaints associated with skin, musculoskeletal and hematologic abnormalities. Some patients also have renal and central nervous system involvement.

The effective management of SLE patients requires regular clinical monitoring in order to assess disease activity and prevent, detect and promptly treat relapses. The prognosis of SLE patients improved in the past decades due to an earlier diagnosis, a more meticulous follow-up and to the use of immunosuppressive drugs to control disease activity. Nevertheless, SLE patients still face noteworthy morbidity and mortality, associated not only with the disease itself, but also with the drugs used for its treatment.

1.2 EPIDEMIOLOGY

Incidence rates of SLE range from 1 to 10 per 100,000 person-years and prevalence rates generally range from 20 to 70 per 100,000 persons (1–13). The annual incidence of juvenile-onset SLE is less than 1 per 100,000 persons (14).

Notably, the SLE epidemiological studies differ not only by sampling and recruitment methodologies, but also by the criteria used to define SLE. Furthermore, the studies conducted at hospital settings failed to capture cases of mild disease. Despite the heterogeneity of the methodology used, there is evidence that the incidence of SLE is increasing (1). Certainly some of this increase is not due to an actual increase in disease occurrence, but the inclusion of milder cases of SLE and the wider availability and use of antinuclear antibody testing.

The strongest risk factor for SLE is gender. It is well known that women are affected more frequently than men. This is an effect partially attributed to estrogens, since in children, in whom sex hormonal levels are minimal, the female-to-male ratio is

3:1, while in women of child-bearing years the ratio ranges from 7:1 to 15:1 (1–13). Furthermore, women with SLE treated with estrogen-containing regimens, such as postmenopausal hormone replacement therapies, have a significantly increased risk of a flare (15).

Even though lupus is less common in men, it is usually more severe (16–18). Men with SLE have higher incidence of skin manifestations, cytopenias, serositis, nephritis, neurologic involvement, cardiovascular disease and vasculitis than women (16–18).

Regarding age, juvenile-onset SLE tends to be more severe, with a higher incidence of nephritis, neurologic involvement, and hematologic abnormalities (19), while lupus tends to be milder in older adults (20). In this group there is a lower incidence of rash, renal and hematologic involvement, while there is a greater prevalence of musculoskeletal manifestations, *sicca* symptoms, serositis and pulmonary involvement (20). Interestingly, in older adults with SLE there is a lower prevalence of hypocomplementemia and of anti-Sm and anti-RNP antibodies and a greater prevalence of rheumatoid factor (20). It has been speculated that older patients may have different genetic determinants of disease and may respond to different triggering mechanisms (19). The senescence of the immune system may also contribute to the clinical differences (19).

SLE has been described all over the world. Ethnicity incorporates genetic, geographic, cultural, social and other characteristics shared within a population. Not surprisingly, the incidence and phenotypic expression of SLE varies between different ethnic groups (1). Prevalence rates in people of African or Asian background are approximately 2 to 3 times higher than in Caucasians (1). In the United States, Americans of European descent have a lower lupus incidence than Asians, Afro-Americans, Afro-Caribbeans, and Hispanic Americans (1).

Social factors certainly determine the prognosis, since it has been consistently shown that the clinical status is poorer in those with less education, lower socioeconomic status and inadequate access to medical care (1).

Geographic factors are also important, since lupus is more common in urban than rural areas (21,22). Some data, however, suggest that rural populations experience poorer clinical outcomes probably related to their low socioeconomic status and educational levels and inadequate access to health care (23). It is well documented that

SLE mortality is lower in large referral hospitals with experience in treating these patients (24).

Another interesting fact is that SLE is rare in Africa, but common in African descendants around the world (1), which shows once again the determinant impact of the environment in this disease.

1.3 CLINICAL MANIFESTATIONS

SLE is a chronic, occasionally life-threatening, disease. The clinical manifestations are diverse, but, usually, the pattern that dominates during the first years of illness tends to prevail throughout its course.

The Euro-lupus cohort is composed of 1,000 unselected SLE patients who have been followed prospectively since 1991 (19). This study determined the incidence of the different clinical manifestations at the onset of the disease and during the follow up and evaluated the response to treatment, damage and mortality. The ultimate goal of the project was to define subsets of patients according to their clinical manifestations and immunological characteristics (19). Data from this study have been extremely important to better understand the clinical manifestations of SLE and will be presented throughout this chapter, complemented by other relevant studies.

1.3.1 Constitutional symptoms

Constitutional symptoms such as fatigue, weight loss and fever are present in the majority of the patients (19). Fatigue is the most common complaint, occurring in more than 80% of patients, and being associated with diminished ability to function (25). Interestingly, depression, pain and poor sleep quality were found to be positive predictors of fatigue in SLE patients (25–27), while disease activity or medication usage were not (26,28). Moreover, perceived social support correlates negatively with fatigue, suggesting a buffering effect (26). Treatment of depression and psychosocial interventions aimed at reducing pain and increasing coping skills are, therefore, of major importance in order to reduce fatigue in this group of patients (26).

1.3.2 Skin and mucosae involvement

Skin involvement is also very frequent. The major categories of cutaneous lupus erythematosus are shown in Table 1.1.

Category	Type of involvement
Acute Cutaneous Lupus Erythematosus	Localized Generalized Toxic epidermal necrolysis-like eruption
Subacute Cutaneous Lupus Erythematosus	Annular Papulosquamous
Chronic Cutaneous Lupus Erythematosus	Localized discoid Generalized discoid Hypertrophic discoid/Verrucous discoid Mucosal discoid Lupus panniculitis/*Lupus profundus* *Lupus tumidus* Chilblains
Other clinical variants	Lupus-lichen planus overlap syndrome Rowell syndrome

Table 1.1 – Major categories of cutaneous lupus erythematosus.

The more classic presentation is the acute cutaneous localized lupus erythematosus, which is characterized by a facial eruption, after sun exposure, that presents in a malar distribution over the cheeks and nose, but sparing the nasolabial folds. It may precede other symptoms of SLE by months or even years. The word “lupus” (Latin for wolf) is attributed to the thirteenth century physician Rogerius who used it to describe the classic malar rash that was reminiscent of a wolf. Despite being the most classic symptom, in the Euro-lupus cohort less than 50% of the patients presented with the typical erythematosus malar rash at the onset of the disease (19).

Subacute cutaneous lupus erythematosus occurs in approximately 10% of SLE patients. The typical lesions are small, erythematous, slightly scaly papules that evolve into either a papulosquamous or annular form, which may coalesce to form polycyclic patterns. The most affected areas are the shoulders, forearms, neck, and upper torso. The face is usually not affected (29).

Chronic discoid lesions occur in up to 25% of SLE patients, but may also occur in the absence of any other SLE features (19). The lesions are usually discrete, erythematous, slightly infiltrated plaques, present mainly on the face, neck, and scalp.

After an active inflammatory phase, the lesions tend to heal, leaving depressed central scars, atrophy, telangiectasias, and hyperpigmentation or hypopigmentation. Scarring alopecia can occur in this group of patients.

Lupus profundus (lupus erythematosus panniculitis) consists of nodules, often painful, which may appear on the scalp, face, arms, chest, back, thighs, and buttocks. They usually resolve, but may leave a depressed area. These nodules consist of fat necrosis with mononuclear cell infiltration and lymphocytic vasculitis.

Chilblain lupus erythematosus presents with tender, red to blue nodules on the toes, fingers, nose, or ears that occur in cold weather and may ulcerate. Approximately 25% of patients who present with this lesion meet SLE criteria (30).

Patients with localized discoid lupus erythematosus, hypertrophic discoid lupus erythematosus, lupus panniculitis, and lupus tumidus tend to have skin disease only. Overall, approximately 19% of the patients with cutaneous lupus erythematosus progress to SLE, with a mean length to progression of 8.2 years (31). The presence of concomitant discoid lupus erythematosus decreases the risk of severe lupus, as these patients have a lower frequency of lupus nephritis, arthritis or pleuritis (32).

Nail lesions, particularly pitting, ridging, and onycholysis, have been identified in 25% of SLE patients (33). Patients with nail changes had a significantly higher incidence of Raynaud's phenomenon and mucous membrane ulcerations (33).

Photosensitivity occurs in 60 to 100% of SLE patients (34). It is common in acute and subacute cutaneous lupus erythematosus and in chronic discoid lupus erythematosus and lupus tumidus. The incidence is greater in those with anti-Ro/SSA antibodies (35).

The majority of SLE patients have hair loss that can lead to areas of alopecia in some patients (36). It can precede other SLE manifestations and may involve the scalp, eyebrows, eyelashes, beard and/or body hair. Alopecia can be nonscarring or scarring. The latter is a complication of discoid lupus erythematosus.

Mucous membrane involvement occurs in 14 to 45% of SLE patients (37–39). Many patients develop oral and/or nasal ulcers, usually painless.

1.3.3 Musculoskeletal manifestations

Arthritis occurs in the majority of SLE patients and is often one of the earliest clinical manifestations (19,40). The arthritis is usually polyarticular and symmetrical, with a predilection for the knees, carpal, metacarpophalangeal and interphalangeal joints. Symptoms in a particular joint can last less than 24 hours. Synovial effusions are infrequent, but when occur they are usually small, and the fluid is clear or slightly cloudy and similar to a transudate (41). The degree of pain often exceeds objective physical findings, and tenderness may be difficult to assess in some patients because of the often coexistence of amplified pain syndrome.

It is typically a non-erosive and non-deforming arthritis, however up to 10% of the patients may show deformities (Jaccoud arthropathy). These may result from soft-tissue abnormalities, such as laxity of ligaments, fibrosis of the capsule, and muscular imbalance. Some patients may show overlapping features with rheumatoid arthritis and develop erosions. The presence of antibodies to citrullinated peptides/proteins (anti-CCP) antibodies, which are present in 8% of SLE patients, is strongly associated with erosive arthritis (odds ratio of 23 for anti-CCP positive versus negative patients) (42).

Myalgias and muscle weakness occur in up to 70% of SLE patients, but atrophy, myositis and severe muscle weakness are relatively uncommon (19). In addition to SLE itself, glucocorticoids and antimalarial drugs can cause muscle weakness without an elevation of the serum levels of creatine kinase and/or aldolase.

1.3.4 Hematologic involvement

Hematologic abnormalities are common among patients with SLE, particularly children. Anemia, defined as a hemoglobin concentration more than two standard deviations below the mean for age and sex, is present in 50% to 75% of children with SLE (43,44). The most common types of anemia in SLE are anemia of chronic diseases, iron deficiency anemia and autoimmune hemolytic anemia. They can occur separately or in combination. Gastrointestinal tract bleeding and lung hemorrhage can also be the cause of anemia and should be considered if there is an elevated reticulocyte count and the direct antiglobulin test is negative.

Leukopenia, defined as a total white blood cell count less than 4,000 /µL (45,46), occurs in nearly two-thirds of children at some point during the course of the illness

(44,47,48). The decrease in the white blood cell count in children with SLE is usually due to a fall in the absolute lymphocyte count. Neutropenia is uncommon and, when it occurs, is usually a drug effect or associated with severe infection.

Significant thrombocytopenia is characterized as a platelet count of less than 100,000/μL in the American College of Rheumatology (ACR) guidelines (45,46). The prevalence of thrombocytopenia varies from 10% to 50% (43,47–49). The degree of thrombocytopenia is usually mild and severe blending is rare (49). The most common clinical manifestations of severe thrombocytopenia are petechiae, purpura and ecchymosis. Epistaxis, gum bleeding, menorrhagia and even intracranial hemorrhage can also occur, but are rare.

Autoimmune hemolytic anemia or immune thrombocytopenia may be the first manifestation of SLE, antedating this diagnosis by many years (50).

Splenomegaly and lymph node enlargement occurs frequently in association with active SLE.

1.3.5 Lupus nephritis

Renal involvement is a significant cause of morbidity and mortality (51). In an American report of 1,378 SLE patients, renal disease (defined as protein excretion >0.5 g/day, ≥5 red blood cells per high-power field, serum creatinine ≥1.5 mg/dL or requiring dialysis or transplant) was present in 32% within one year of diagnosis (52). The incidence of lupus nephritis is higher in African-Americans (34% to 51%), Hispanics (31% to 43%), and Asians (33% to 55%) than it is in Caucasians (14% to 23%) (53–55). Moreover, African-Americans and Hispanics present more frequently with severe underlying histopathology, higher serum creatinine concentrations, and more proteinuria (56). In the Euro-lupus cohort nephritis occurred in 28% of patients (19), a lower incidence when compared with American series (40,52) and Asian series (57). These differences may reflect not only genetic, but also environmental factors.

Most renal abnormalities occur within the first 6 to 36 months (53). The patients may present with asymptomatic hematuria and/or proteinuria, nephrotic syndrome or rapidly progressive glomerulonephritis with loss of renal function.

Several forms of glomerulonephritis can occur and renal biopsy is useful to define the type and extent of the disease.

In addition to the glomerulopathies, there are other forms of lupus renal disease, namely tubulointerstitial nephritis and vascular disease.

Tubulointerstitial disease is common in lupus nephritis and is almost always associated with concurrent glomerular disease. Patients present with a rising plasma creatinine concentration and a relatively bland urinalysis. Signs of tubular dysfunction may be present. The severity of the tubulointerstitial involvement is an important prognostic sign, correlating positively with the presence of hypertension, an elevated plasma creatinine concentration, and a progressive clinical course (58).

Involvement of the renal vasculature is also frequent in lupus nephritis, and it can also adversely affect the prognosis (59–61). The most frequent manifestations are thrombotic microangiopathy, vasculitis and atherosclerosis (59–61). Rarely do patients with lupus nephritis develop renal vein thrombosis. These patients are typically nephrotic and have high antiphospholipid antibody levels.

Finally, regarding lupus nephritis outcome, in a large, recent, multi-ethnic inception cohort of 1,827 SLE patients, of whom 700 (38.3%) had lupus nephritis, the estimated overall 10-year incidence of end stage renal disease was 4.3% (95% CI: 2.8%, 5.8%) (51). Patients with nephritis had a higher risk of death (HR = 2.98, 95% CI: 1.48, 5.99; p= 0.002) (51). In conclusion, despite the current standard of care, nephritis is still associated with end stage renal disease and death, defining the prognosis of SLE patients (51). During my Ph.D. I chose, therefore, to focus on this important manifestation of SLE.

1.3.6 Neuropsychiatric involvement

Neuropsychiatric involvement of SLE consists of a broad range of manifestations, which are shown in Table 1.2.

Cognitive dysfunction, expressed by impairment in memory, abstract thinking and judgment is very prevalent in SLE (62–64). In a study comparing SLE patients and normal controls, patients were more cognitively impaired, particularly on tests emphasizing psychomotor speed, complex attention, and memory (65). Other previous studies of cognitive impairment in SLE suggest that the deficits most typically involve attention, free recall of recently-learned information, information processing speed, working memory, and spatial processing (66). It is not uncommon for adolescents with SLE to present with progressive deterioration in academic performance, depression, and social isolation.

Central	Peripheral
Psychosis	Mononeuropathy
Mood disorder	Cranial neuropathy
Anxiety disorder	Polyneuropathy
Acute confusional state	Plexopathy
Cognitive dysfunction	Autonomic neuropathy
Headache	Guillan-Barré syndrome
Movement disorder	*Myasthenia gravis*
Seizures	
Demyelinating syndrome	
Myelopathy	
Aseptic meningitis	
Cerebrovascular disease	

Table 1.2 – **Neuropsychiatric manifestations in SLE.**

Headache is also a common complaint of SLE patients, with more than 50% of patients reporting headache (migraine 32% and tension-type headache 24%) (67). Nevertheless, the prevalence of headache is not different from controls (67). No particular pathogenetic mechanism of headache in SLE patients has been identified, nor has been established an association between headache and the disease status (67).

Seizures occur in 5% to 12% of SLE patients (68–70). Both generalized and partial seizures can occur. Complex partial seizures are more common than primary generalized seizures, although the latter tend to be more frequent in patients with lupus nephritis and hypertension. Seizures may be the first manifestation of lupus or may develop during the course of the illness, usually during the first year after diagnosis (68–70). The causes of seizures are varied and may reflect an acute inflammatory episode or chronic central nervous system damage. Other contributing factors are hypertension, stroke, metabolic disturbances, infections and tumors (70).

Approximately 15% of SLE patients develop a peripheral neuropathy due to vasculopathy of small arteries supplying the nerves (71). Polyneuropathy is the most frequent (56%), followed by cranial neuropathy (13%), mononeuropathy (11%), and mononeuritis multiplex (9%)(71). Peripheral neuropathy due to SLE is usually asymmetric and mild, affecting mainly sensory nerves. Paresthesias and numbness of the digits that are often worse at night are a common type of presentation.

Autonomic neuropathy has also been reported, resulting in gastrointestinal, bladder, cardiac, pupillary, and sweating abnormalities (72). In contrast to seizures,

peripheral neuropathy does not typically occur early in the course of SLE. Cranial neuropathies have also been described, causing diplopia, nystagmus, ptosis, visual field deficits, trigeminal neuralgia, dysarthria, facial weakness or vertigo (73).

Optic neuritis and sensorioneural hearing defects in both low and high frequencies have been noted more frequently in patients with SLE than in the general population (74,75).

Transverse myelitis has also been described in SLE patients presenting with the sudden onset of lower extremity weakness and/or sensory loss, plus loss of rectal and urinary bladder sphincter control (76). Arteritis, with resultant ischemic necrosis of the spinal cord, is thought to be the cause of transverse myelitis in SLE.

1.3.7 Pulmonary manifestations

Pleuritis is the most frequent pulmonary manifestation of SLE, but pneumonitis, interstitial lung disease, pulmonary hypertension, shrinking lung syndrome, and alveolar hemorrhage can also occur.

Subclinical lung disease, including restrictive lung disease or reduced diffusion capacity identified through pulmonary function testing with diffusing capacity for carbon monoxide, is present in 60% to 70% of patients (77–79).

1.3.8 Cardiovascular involvement

The heart is one of the most frequently affected organs in SLE (80). Any part of the heart can be affected, including the pericardium, myocardium, endocardium, coronary arteries, valves and the conduction system.

Pericarditis, with or without an effusion, is the most common cardiac manifestation of SLE, occurring in approximately 25% of patients at some point during their disease course (80). Myocarditis is uncommon, but may be severe and cause congestive heart failure (80). Libman-Sacks endocarditis is usually clinically silent, but it can produce valvular insufficiency, be a source of emboli and contribute to the occurrence of subacute bacterial endocarditis.

Patients with SLE also have an increased risk of coronary artery disease, an important cause of mortality in older adult patients and in those with long-standing SLE (80). In one study, 5 of 40 children with SLE without symptoms or history of ischemic

heart disease had abnormal coronary perfusion (81), which shows ischemic heart disease starts subclinically very early in this group of patients.

SLE is also associated with stroke and premature death due to cerebrovascular disease (82,83). SLE is a risk factor for all stroke subtypes, except for subarachnoid hemorrhage (84). Strokes can be particularly severe in this setting (85). In a prospective cohort, 77% of strokes were classified as National Institute of Health (NIH) stroke scale of >6 (85). In another observational study of 2,688 Canadian SLE patients, the risk of death due to stroke or other cerebrovascular disease was doubled when compared with non-SLE controls (83). Baseline disease activity, hyperlipidemia, and hypertension are the main risk factors (85). There is also a high association between antiphospholipid antibodies and stroke in SLE (86). Other factors, such as vasculitis, valvular heart disease, infection, chronic steroid therapy, emboli, and/or thrombosis, can also contribute cerebrovascular disease (87,88).

Vasculopathy can also be seen in SLE. Raynaud phenomenon, a vasospastic process induced by cold or emotion, occurs in up to 50% of patients (19). Vasculitis prevalence ranges between 11 and 36% (89). Vessels of all sizes can be involved, with cutaneous small vessel vasculitis being the most common. The latter can manifest as palpable purpura, petechiae, livedo reticularis, panniculitis and ulcerations. Livedo reticularis, which is caused by vasospasm of the dermal ascending arterioles, can progress to vascular occlusion, leading to ischemia and to tissue infarction, causing painful ulcerations.

Medium and large vessel vasculitis can also occur and cause life-threatening manifestations such as mesenteric vasculitis, coronary vasculitis, pulmonary bleeding, retinal vasculitis or peripheral or central nervous system vasculitis (89).

Thromboembolic disease can complicate SLE, particularly in the context of antiphospholipid syndrome. In a large cohort of 554 newly-diagnosed SLE patients followed for a median of 6.3 years, an arterial thrombotic event occurred in 11%, whereas a venous thrombotic event occurred in 5% (90). Arterial thromboemboli may cause focal neurologic problems, such as stroke, seizures, or more diffuse cognitive defects.

1.3.9 Ocular manifestations

SLE patients can also complain of ocular symptoms, which correlate with disease activity and can be the initial manifestation of SLE (91). Keratoconjunctivitis *sicca* associated with secondary Sjögren's syndrome and retinal vasculopathy are the most common ocular manifestations (91). Although less frequently, the anterior segment of the eye can also be involved, occurring anterior uveitis, keratitis and episcleritis. Eyelids can also be affected by discoid lupus. In addition, several drugs used for SLE treatment are associated with ocular toxicity including glucocorticoids, which cause glaucoma and cataracts, and antimalarial agents that can cause retinal toxicity (91). Vision loss may result from involvement of the retina, choroid and optic nerve (91).

1.3.10 Gastrointestinal involvement

SLE-related gastrointestinal abnormalities occur in up to 40% of patients during their lifetime and can involve almost any organ along the gastrointestinal tract and include esophagitis, peptic ulcer disease, intestinal pseudo-obstruction, protein-losing enteropathy, mesenteric vasculitis, and peritonitis (92).

Intestinal pseudo-obstruction is rare and is characterized by abdominal pain, bloating, and distension in the absence of an anatomic lesion obstructing the intestine. It usually occurs in SLE patients with active disease (93). It can be caused by immune complex deposition in smooth muscle cells, and/or vasculitis causing chronic ischemia and hypomotility (94,95).

Mesenteric vasculitis is also rare. It can present insidiously with postprandial abdominal pain, weight loss, nausea, vomiting, and diarrhea or acutely due to infarction, perforation, and peritonitis (96).

Primary peritonitis secondary to SLE can develop acutely during a lupus flare and cause intense abdominal pain or chronically presenting with a gradually developing, painless, ascites (97).

Liver test abnormalities in SLE patients are common, but clinically significant liver disease is rare (98,99). Drug-induced damage, steatosis, vascular thrombosis, overlap with autoimmune hepatitis, or SLE itself are the main causes of liver disease in this group of patients (99).

Finally, acute pancreatitis occurs in 2% to 8% of SLE patients (100), most frequently when the disease is active.

1.4 AUTO-ANTIBODIES

Immunologic abnormalities, particularly the production of autoantibodies, are frequent in SLE. The presence of antinuclear antibodies in the serum is seen in the great majority of SLE patients. Occasional cases of SLE patients without detected anti-nuclear antibodies have been described (101), but alternative diagnoses should be sought vigorously in these cases.

Interestingly, autoantibodies are typically present many years before the diagnosis of SLE, with a progressive accumulation of specific autoantibodies while the patients are still asymptomatic (102).

Anti-dsDNA antibodies are detected in approximately 70% of SLE patients. A high titer of anti-dsDNA antibodies is one of the best markers of SLE activity (19). Moreover, it is associated with a higher prevalence of nephropathy, hemolytic anemia and fever (19).

Anti-Smith (anti-Sm) and anti-ribosomal P protein antibodies are also highly specific for SLE, but lack sensitivity, being present in less than 10%-30% of SLE patients (19,103,104). Anti-ribosomal P antibodies are associated with active disease, particularly neuropsychiatric, renal, or hepatic involvement, and are found more frequently in children than adults with SLE (105,106).

Anti-Ro/SSA antibodies occur in 20% to 30% of patients and are associated with subacute cutaneous lesions, *sicca* syndrome and a lower prevalence of thrombocytopenia (19). Anti-La/SSB antibodies are present in 20% of SLE patients and are associated with malar rash, subacute cutaneous lesions, photosensitivity, arthritis, serositis and thrombosis (19).

Anti-U1-snRNP antibodies are detected in 13% to 25% of patients (19,104). Patients with these antibodies have a higher incidence of Raynaud's phenomenon, myositis and lymphadenopathy (19).

The presence of anti-Ro/SSA, anti-La/SSB and anti-U1-snRNP during pregnancy is associated with neonatal lupus (107–109). These antibodies cross the placenta and accumulate mainly on the cardiac tissue of the phetus, where they may contribute to the

degeneration of the cardiac electric conduction system. Neonatal lupus is, indeed, the cause of 80% to 95% of the cases of complete atrioventricular block diagnosed in uterus or in the neonatal period (107). Other possible clinical manifestations of neonatal lupus are rash, hematologic abnormalities, and hepatic abnormalities, including asymptomatic elevation of the transaminases, hepatosplenomegaly, hepatitis and cholestasis (108). Aproximately half of the mothers of newborns with neonatal lupus are asymptomatic (108). The others have been diagnosed previously with SLE, Sjögren disease or mixed connective tissue disease (108).

Antiphospholipid antibodies (lupus anticoagulant, IgG and IgM anticardiolipin antibodies; and IgG, IgM and Ig A anti-β2-glycoprotein I) should also be mentioned. These are strongly associated with thrombosis, spontaneous fetal losses and thrombocytopenia (19).

Finally, in two small case series, antibodies to microtubule-associated protein 2 were found in a high percentage (63% to 76%) of SLE patients with neuropsychiatric manifestations and not in patients with other neurologic diseases, SLE patients without neurolupus or healthy controls (110,111).

In conclusion, SLE patients might have different auto-antibodies, which are associated with different clinical manifestations. Nevertheless, a perfect distinction of SLE subsets according to the profile of auto-antibodies is not possible. We need, therefore, new biomarkers to distinguish the different subtypes of SLE. A better understanding of the pathogenesis of the disease might be helpful to reach this goal and this was one of the aims of my PhD.

1.5 DIAGNOSIS

1.5.1 Diagnosis of SLE

The clinical heterogeneity of SLE and the lack of pathognomonic features or tests pose a diagnostic challenge, which is reflected in the long delay between the onset of symptoms and the diagnosis (2 years in adults and 5 years in children) (19). The diagnosis of SLE is generally based on clinical judgment, after excluding alternative diagnoses. Classification criteria have been developed as a means of identifying SLE patients for

study purposes. These criteria have been useful also for clinicians as a diagnostic tool. In 1982, the American Rheumatism Association (ARA), now the American College of Rheumatology (ACR), developed an SLE criteria classification, which was revised in 1997 (45,46). The patient was diagnosed with SLE if four or more of the manifestations were present, either serially or simultaneously (46,112). The ACR criteria are presented in Table 1.3.

In 2012, the Systemic Lupus International Collaborating Clinics (SLICC) proposed revised SLE criteria that were developed to address some of weaknesses of the 1997 ACR SLE criteria (113). In order to be diagnosed with SLE the patient has to satisfy at least four of 17 criteria, including at least one of the 11 clinical criteria and one of the six immunologic criteria, or the patient has to have biopsy-proven lupus nephritis in the presence of antinuclear antibodies or anti-dsDNA antibodies (113). The SLICC criteria are shown in Table 1.4.

The SLICC criteria were derived from a set of 702 expert-rated scenarios and validated in a new sample of 690 new patient scenarios (113). The new criteria had greater sensitivity (94% versus 86%; $p<0.0001$) and equal specificity (92% versus 93%; $p=0.39$) when compared to the 1997 ACR criteria (113).

The SLICC criteria include a more diverse group of skin and neuropsychiatric manifestations and, very importantly, include low complement and anti-β 2-glycoprotein I antibody (IgA, IgG, or IgM) as immunologic criteria.

In a study in which patients were grouped according to whether the SLICC criteria were met before, at the same time as, or after the ACR criteria, out of 622 patients, 319 (50%) were classified at the same time, 78 (12%) earlier and 225 (35%) later (mean 4.4 years) (114). Among the patients diagnosed later with the SLICC criteria, in the majority of cases the delay was due to malar rash and photosensitivity counting only as one criterion (acute cutaneous SLE) in the SLICC classification, unstead of two criteria in the ACR classification.

Criterion	Definition
Malar rash	Fixed erythema, flat or raised, over the malar eminences, sparing the nasolabial folds
Photosensitivity	Skin rash as a result of unusual reaction to sunlight
Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging
Oral ulcers	Oral or nasopharyngeal ulcers, usually painless
Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion
Serositis	Pleuritis – pleuritic pain or rubbing detected by a clinician or evidence of pleural effusion or Pericarditis – documented by electrocardiogram, rub or evidence of pericardial effusion
Renal disorder	Persistent proteinuria greater than 500mg/24h or greater than 3+ if quantification not performed or Cellular casts – may be red cell, hemoglobin, granular, tubular, or mixed
Neurologic disorder	Seizures or psychosis, in the absence of drugs or known metabolic conditions
Hematologic disorder	Hemolytic anemia or Leukopenia – less than 4,000/μL on two or more occasions or Lymphopenia – less than 1,500/μL on two or more occasions or Thrombocytopenia – less than 100,000/μL, in the absence of drugs
Antinuclear antibodies	An abnormal titer of antinuclear antibodies by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs
Immunologic disorders	Anti-DNA – Antibody to native DNA in abnormal titer or Anti-Sm – Presence of antibody to Sm nuclear antigen or Positive finding of antiphospholipid antibody – Abnormal serum level of IgG or IgM anticardiolipin antibodies or a positive test result for lupus anticoagulant, or a false-positive serologic test for syphilis known to be positive for at least six months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test

Table 1.3 – ACR Criteria for the diagnosis of SLE (112).

Criterion	Definition
Clinical Criteria	
Acute or subacute cutaneous lupus	Acute cutaneous lupus Lupus malar rash Bullous lupus Toxic epidermal necrolysis variant of SLE Maculopapular lupus rash Photosensitive lupus rash (in the absence of dermatomyositis)
	Subacute cutaneous lupus Nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasias
Chronic cutaneous lupus	Classic discoid rash Hypertrophic (verrucous) lupus Lupus panniculitis (profundus) Mucosal lupus Lupus erythematosus tumidus Chilblains lupus or Discoid lupus/lichen planus overlap
Oral ulcers	Palate, buccal, tongue or nasal ulcers, in the absence of other causes, such as vasculitis, Behçet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis, and acidic foods
Nonscarring alopecia	Diffuse thinning or hair fragility with visible broken hairs in the absence of other causes, such as alopecia areata, drugs, iron deficiency, and androgenic alopecia
Joint disease	Synovitis involving two or more joints, characterized by swelling or effusion or tenderness in two or more joints and at least 30 minutes of morning stiffness
Serositis	Typical pleurisy for more than one day, Pleural effusions or Pleural rub
	Typical pericardial pain (pain with recumbency improved by sitting forward) for more than one day, Pericardial effusion, Pericardial rub, or Pericarditis by electrocardiography in the absence of other causes, such as infection, uremia and Dressler syndrome
Renal disorder	Urine protein-to-creatinine ratio or 24-hour urine protein representing 500 mg protein/24h or Red blood cell casts
Neurologic disorder	Seizures Psychosis Mononeuritis multiplex, in the absence of other known causes, such as primary vasculitis Myelitis Peripheral or cranial neuropathy, in the absence of other known causes, such as primary vasculitis, infection, and diabetes mellitus or

	Acute confusional state, in the absence of other causes, including toxic/metabolic, uremia and drugs
Hemolytic anemia	Hemolytic anemia
Leukopenia or lymphopenia	Leukopenia (<4,000/μL at least once), in the absence of other known causes, such as Felty syndrome, drugs, and portal hypertension or Lymphopenia (<1,000/μL at least once), in the absence of other known causes, such as glucocorticoids, drugs, and infection
Thrombocytopenia	Thrombocytopenia (<100,000/μL) at least once in the absence of other known causes, such as drugs, portal hypertension, and thrombotic thrombocytopenic purpura
Immunologic Criteria	
Antinuclear antibodies	Antinuclear antibodies levels above laboratory reference range
Anti-dsDNA	Anti-dsDNA antibody level above laboratory reference range (or more than twofold the reference range if tested by ELISA)
Anti-Sm	Presence of anti-Sm antibody
Antiphospholipid antibody	Lupus anticoagulant Medium or high titer anticardiolipin antibody level (IgA, IgG, or IgM) Anti-β 2-glycoprotein I antibody (IgA, IgG, or IgM) or False-positive test result for rapid plasma reagin
Low complement	Low C3, low C4 or low CH50
Direct Coombs test	Direct Coombs test in the absence of hemolytic anemia

Table 1.4 – The 2012 Systemic Lupus International Collaborating Clinics (SLICC) proposed revised criteria (113).

1.5.2 Diagnosis of lupus nephritis

In order to promptly diagnose lupus nephritis, SLE patients should undergo testing at regular intervals, including a urinalysis with examination of the urinary sediment, a spot urine protein-to-creatinine ratio, and a serum creatinine.

A kidney biopsy should be performed in SLE patients who develop evidence of renal involvement in order to establish the diagnosis, including patients with acute increase in serum creatinine, proteinuria higher than 500mg/24h or urine protein/creatinine higher than 0.5 g protein/ g creatinine, hematuria in presence of any level of proteinuria, and active sediment/cellular casts (115).

Several studies have illustrated the lack of reliability of diagnoses rendered on the basis of clinical features alone. Firstly, the kidney biopsy differentiates lupus nephritis from other non-lupus kidney diseases that may also affect SLE patients, including, for instance, drug-induced interstitial nephritis or renal thrombotic microangiopathy.

Secondly, if lupus nephritis is indeed identified, the kidney biopsy is also important to classify the type of lesions and to guide the treatment strategies used. Determining the diagnosis based only on the clinical presentation may be difficult, particularly in patients with low or moderate levels of proteinuria and without acute kidney injury. Such a presentation could be the result of mesangial lupus, a mild membranous lupus or a proliferative lesion, either with mild activity or in the early stages of a more active lesion. Finally, with improved imaging and the use of semi-automated biopsy guns, kidney biopsy associated complications are uncommon. Overall, the kidney biopsy is, therefore, a valuable instrument to guide the clinical approach of an SLE patient who presents with urine analysis changes.

Most patients with lupus nephritis have an immune complex-mediated glomerular disease. IgG glomerular deposits and co-deposits of IgA, IgM, C3, and C1q, the so-called "full house" immunofluorescence pattern, are frequent in lupus nephritis. The glomerular deposits can be identified in the mesangial, subendothelial, and subepithelial locations. Extraglomerular immune deposits can also be seen the interstitium and blood vessels.

Determining the class of lupus nephritis is important, since treatment is guided by the histologic subtype and the clinical presentation may not accurately reflect the severity of the histologic findings, as previously discussed. Numerous serum and urine markers have been studied as potential noninvasive determinants of lupus nephritis stages (116), including anti-C1q antibodies (117), tumor necrosis factor-like weak inducer of apoptosis (TWEAK) (118), and urinary neutrophil gelatinase-associated lipocalin (NGAL) (119). Nevertheless, currently no marker of disease activity provides the degree of information that is gained by histopathology.

The 2004 Renal Pathology Society/International Society of Nephrology classification divides SLE glomerular disorders into six different classes based upon kidney biopsy histopathology (120,121):

- **Class I (Minimal mesangial lupus nephritis)** — It is characterized by the inexistence of abnormalities on light microscopic. Mesangial immune deposits are identified by immunofluorescence and electron microscopy. This is the earliest and

mildest form of glomerular involvement in SLE. Patients typically have a normal urinalysis, or minimal proteinuria, and a normal serum creatinine.

- **Class II (Mesangial proliferative lupus nephritis)** — Mesangial hypercellularity and mesangial matrix expansion are the main features of this class. A few isolated subepithelial or subendothelial deposits may be seen on immunofluorescence or electron microscopy. The usual clinical manifestations are microscopic hematuria and/or proteinuria.

- **Class III (Focal lupus nephritis)** - It is defined histologically by endocapillary or extracapillary glomerulonephritis in less than 50% of glomeruli studied by light microscopy. Lesions are almost always segmental, involving less than 50% of the glomerular tuft. Electron microscopy usually reveals immune deposits in the mesangium and subendothelial space of the glomerular capillary wall. Class III is subdivided according to the inflammatory activity and chronicity of the lesions:

  - Class III (A) – It represents class III disease with active lesions. It is also referred to as focal proliferative lupus nephritis.

  - Class III (A/C) – It is characterized by active and chronic lesions. It is also called focal proliferative and sclerosing lupus nephritis.

  - Class III (C) – It is identified by chronic inactive lesions with scarring. This is also called focal sclerosing lupus nephritis.

  Patients usually have hematuria and proteinuria, and some patients have hypertension, a decreased glomerular filtration rate, and/or nephrotic syndrome.

- **Class IV (Diffuse lupus nephritis)** – It is the most common and severe form of lupus nephritis. It is defined by proliferative and necrotizing lesions and crescents in more than 50% of glomeruli. The marked deposition of immunoglobulins, particularly IgG, and complement results in thickening of the glomerular capillary wall, causing a pattern similar to a membranoproliferative glomerulonephritis. The lesions may be segmental (class IV-S, involving less than 50% of the glomerular tuft) or global (class IV-G, involving more than 50% of the glomerular tuft). The presence of diffuse wire loop deposits, with little or no glomerular proliferation, is also considered class IV disease. There are also subclasses of class IV lupus nephritis that are determined by the inflammatory activity or chronicity of the lesions, like it was described for class III lupus nephritis. All patients have hematuria and proteinuria, and nephrotic syndrome,

hypertension, and reduced glomerular filtration rate are frequently seen. Affected patients typically have significant hypocomplementemia, particularly C3, and elevated anti-dsDNA antibodies levels.

- **Class V (Lupus membranous nephropathy)** – It is characterized by diffuse thickening of the glomerular capillary wall on light microscopy and by subepithelial immune deposits on immunofluorescence or electron microscopy. If subendothelial deposits are also identified by immunofluorescence or electron microscopy a combined diagnosis of classes III and V disease, or of classes IV and V disease should be done, based upon the distribution of the deposits. Class V patients typically present with signs of the nephrotic syndrome. Microscopic hematuria and hypertension may also be seen at presentation, and the creatinine concentration is usually normal or only slightly elevated.
- **Class VI (Advanced sclerosing lupus nephritis)** – It is characterized by global sclerosis of more than 90% of glomeruli, without active glomerulonephritis. It represents the advanced stage of chronic class III, IV, or V lupus nephritis. Patients usually have proteinuria and a slowly progressive renal dysfunction. Immunosuppressive therapy is unlikely to be beneficial.

The presence and severity of tubulointerstitial and vascular involvement should be noted in any biopsy specimen. Additional histologic features should also be included in the description, namely the proportion of glomeruli affected by fibrinoid necrosis and crescents.

Other variants of kidney disease can also be seen in patients with SLE, including collapsing glomerulosclerosis and lupus podocytopathy. The former is characterized by the collapse of the glomerular capillary tuft with epithelial cell proliferation in the Bowman space. The patients usually have nephrotic syndrome, renal impairment, and rapid progression to end-stage renal disease (122). Lupus podocytopathy is defined by diffuse epithelial cell foot process effacement without immune complex deposition, similar to what happens in minimal changes disease (123,124). It can be caused by cytokines toxic to podocytes, or podocyte injury driven by T cell dysfunction or drugs (123–125).

The International Society of Nephrology /Renal Pathology Society appears to provide increased reproducibility compared to the modified 1982 World Health Organization (WHO) system (126,127). Nevertheless, this system is still far from ideal,

since it does not predict treatment response or prognosis. Furthermore, a significant percentage of patients evolve from one class of lupus nephritis to another, sometimes after therapy and sometimes spontaneously. Finally, the relatively small number of glomeruli that are obtained on a typical percutaneous renal biopsy may cause a sampling error, which may lead to an erroneous characterization of the percentage of glomeruli involved by the disease and consequently an erroneous evaluation of the lupus nephritis class and prognosis.

Recently, it was developed a renal activity index for lupus (RAIL) based exclusively on laboratory measures that accurately reflects the histologic lupus nephritis activity (128). The variables used were the urinary levels of NGAL, monocyte chemoattractant protein 1 (MCP1), ceruloplasmin, adiponectin, hemopexin, and kidney injury molecule 1 standardized by urine creatinine (128). The RAIL quantifies the amount of histologic inflammation seen on the tissues, as measured by the NIH activity index with over 92% accuracy (128). Furthermore, the RAIL is minimally influenced by concurrent lupus nephritis chronicity and reflects both glomerular and tubulointerstitial inflammation (128). The concurrent use of medications, including drugs targeting the renin-angiotensin-aldosterone system, does not decrease the accuracy of this index (128). This index seems promising, but needs to be further validated.

In conclusion, new, non-invasive, methods to diagnose lupus nephritis and to evaluate its activity and response to treatment are much needed. During my Ph.D. one of my goals was precisely to contribute to the quest of a clinically useful biomarker for lupus nephritis. I developed a research project that allowed me to identify a new pathway responsible for the control of mesangial cell proliferation in the kidneys of patients with lupus nephritis. This work made possible the identification of a novel urinary marker of lupus nephritis activity: HER2. This is a promising finding considering that it establishes strong foundations not only for a new diagnostic approach, but also for new treatment strategies.

1.5.3 Diagnosis of neuropsychiatric lupus

Regarding neurolupus, its diagnosis is also challenging, since it can mimic symptoms due to intercurrent illness, medication use and functional

disturbances. Psychometric testing and psychiatric interviews can be helpful to differentiate functional from organic disease.

Routine evaluation of the cerebrospinal fluid may be normal, except in cases of aseptic meningitis, vasculitis, and transverse myelitis.

Computerized tomography (CT) scans and magnetic resonance imaging (MRI) are useful for detecting structural abnormalities. MRI is more sensitive than CT, and may reveal changes that reflect focal neuropsychiatric lupus. Acute, reversible lesions are characterized by the lack of discrete borders, intermediate intensity on T2-weighted images, and overlying or adjacent gray matter hyperintensity (129). Nevertheless, the diagnosis of neurolupus is difficult by MRI since white matter lesions and periventricular hyperintensities are also detected in SLE patients without neurolupus and even in healthy individuals (130).

SLE patients have abnormalities of cell metabolism and regional blood flow detectable by positron emission tomography (PET) (131,132). This test is expensive and provides scarce additional diagnostic information beyond that obtained with MRI, so it is only used exceptionally.

Angiography has limited sensitivity for detecting the small vessel disease characteristic of SLE. Magnetic resonance angiography is also unable to accurately demonstrate these lesions (129).

1.6 DIFFERENTIAL DIAGNOSIS

Several infections, malignancies and other autoimmune diseases can cause clinical manifestations similar to SLE. Cytomegalovirus and Epstein-Barr virus infections, for instance, can cause fever, fatigue, lymph node enlargement and hepatosplenomegaly. In addition, Epstein-Barr infection may lead to positive antinuclear antibodies and positive anti-dsDNA antibodies. Human parvovirus B19 can also cause flu-like symptoms, arthritis and hematologic abnormalities such as leukopenia and thrombocytopenia, which can be observed in SLE. Human immunodeficiency virus (HIV), hepatitis B virus and hepatitis C virus can also cause a similar clinical presentation. Some

bacterial infections, including salmonellosis and tuberculosis, should also be considered in the differential diagnosis.

Leukemias, lymphomas and myelodysplastic syndromes may present with constitutional symptoms, hematologic abnormalities, splenomegaly, lymphadenopathy and increased lactate dehydrogenase (LDH) levels, similar to what happens in SLE. Monoclonal expansion of B and T cells, monocytosis, and macrocytosis can help to distinguish these malignancies from SLE.

Patients with thrombotic thrombocytopenia purpura may have fever and thrombocytopenia, common manifestations in SLE patients. Microangiopathic hemolytic anemia, acute kidney injury, fluctuating neurological manifestations, and/or low levels of ADAMTS13 are more characteristic of thrombotic thrombocytopenia purpura.

Other autoimmune diseases may have similar clinical manifestations, which may cause diagnostic difficulties. Early rheumatoid arthritis, for instance, may be difficult to distinguish from SLE, since both conditions can cause arthritis, fatigue, *sicca* symptoms, subcutaneous nodules, serositis and anemia. Features of later stages of rheumatoid arthritis, including swan neck deformities, ulnar deviation and soft tissue laxity, can also be observed in some SLE patients who present with Jaccoud arthropathy. Furthermore, antinuclear antibodies may be positive in up to one-half of patients with rheumatoid arthritis and rheumatoid factor may be present in approximately one-third of SLE patients. Some clinical features and laboratory tests, however, may help distinguish the two diseases. The joint deformities in SLE, for instance, are often reducible and infrequently erosive and the presence of anti-CCP antibodies is more supportive of the diagnosis of rheumatoid arthritis.

Systemic Juvenile Idiopathic Arthritis (sJIA) and adult Still's disease can cause fever, arthritis, rash, lymph node enlargement and hepatosplenomegaly. Patients typically do not have antinuclear antibodies and often present with leukocytosis rather than leukopenia.

Patients with Sjögren syndrome may have extraglandular manifestations, such as neurologic and pulmonary abnormalities, and the presence of antinuclear antibodies, which may mimic SLE. Keratoconjunctivitis *sicca*, xerostomia, characteristic findings on salivary gland biopsy and anti-SSA/Ro and anti-SSB/La antibodies are associated with Sjögren syndrome, but may also occur in SLE patients, who may have the two conditions.

Mixed connective tissue disease has overlapping features of SLE, systemic sclerosis, and polymyositis. The features occur sequentially, often over a period of years, which makes the diagnosis challenging. High titers of anti-U1 RNP antibodies are frequent.

Patients with systemic sclerosis can also present with analogous clinical features. Furthermore, antinuclear antibodies are present in most patients. Anti-dsDNA and anti-Sm antibodies, however, are not commonly observed in systemic sclerosis patients. These express more frequently antibodies anti-Scl-70 (topoisomerase I) or antibodies to centromere proteins. In addition, sclerodactyly, telangiectasias, calcinosis, and malignant hypertension with acute kidney injury are more consistent with systemic sclerosis.

Dermatomyositis and polymyositis should also be considered in the differential diagnosis. SLE patients can present with low-grade myositis, but usually it is less severe than the overt proximal muscle weakness characteristic of dermatomyositis and polymyositis. The cutaneous manifestations are also fairly different. Gottron papules, a heliotrope eruption and photodistributed poikiloderma are characteristic of dermatomyositis. On the other hand, nephritis and hematologic abnormalities are absent in dermatomyositis and polymyositis. Antinuclear antibodies are identified in 30% of dermatomyositis and polymyositis patients, compared with almost all patients in SLE. The former may also express myositis-specific antibodies such as anti-Jo-1.

Patients with medium and small vessel vasculitides such as polyarteritis nodosa, granulomatosis with polyangiitis, microscopic polyangiitis and Behçet disease may present with constitutional symptoms, skin lesions, neuropathy and renal dysfunction. Usually these patients do not have antinuclear antibodies and renal lesions do not have immune deposits.

Patients with undifferentiated connective tissue disease have clinical manifestations suggestive of a systemic autoimmune disease, but do not satisfy the criteria for a defined disease. The majority of these patients maintains an undefined profile and has a mild disease course, but a few may progress to SLE (133).

Finally, Kikuchi-Fujimoto disease can also present in a similar way. This is a benign form of histiocytic-necrotizing lymphadenitis, which may cause fever, myalgias, arthralgias, lymphadenopathy and hepatosplenomegaly. It is usually a self-limited condition, with spontaneous remission occurring within a few months.

In conclusion, since SLE can cause a myriad of clinical manifestations, its differential diagnosis is vast and includes infectious diseases, malignancies and other autoimmune diseases.

1.7 PARTICULARITIES OF JUVENILE-ONSET SLE

Juvenile-onset SLE is fundamentally the same disease as adult-onset SLE, with globally similar etiology, pathogenesis, clinical manifestations and laboratory findings. It is well known, however, that age at disease onset has an impact on the clinical course and outcome of the disease (19). Furthermore, the care of children and adolescents with SLE is different from that of adults because of the physical and psychologic impact of the disease and its treatment on growth and development.

The presenting manifestations of SLE in children are as diverse as they are in adults. Some children present with low-grade fever, weight loss and malaise with general deterioration over several months, while others present with acute or life-threatening events. As previously discussed, patients with juvenile-onset SLE compared to adults with SLE more often have severe organ involvement as a presenting manifestation, particularly nephritis (19).

Table 1.5 shows the most common presenting manifestations in juvenile-onset SLE, according to two studies performed in a French (134) and in a Canadian cohort (135).

Clinical Features	French cohort (%)	Canadian cohort (%)
Hematologic	72	55
Anemia	27	NA
Leukopenia	35	NA
Lymphopenia	NA	29
Thrombocytopenia	28	29
Mucocutaneous	70	NA
Musculoskeletal	64	61
Fever	58	39
Renal	50	NA

Table 1.5 – **Frequency of presenting clinical features in juvenile-onset SLE** (NA – data not available)**.**

Globally, fever, nephritis, neurologic involvement, thrombocytopenia and hemolytic anemia are more common in juvenile-onset SLE, whereas Raynaud's phenomenon, pleuritis and *sicca* symptoms are more common in adult onset SLE (19,136).

The diagnosis of juvenile-onset SLE is usually difficult, because the most typical signs and symptoms are less common and doctors are reluctant to diagnose SLE in childhood. In the Euro-lupus cohort, 76 out of the 1,000 SLE patients (8%) developed the disease before the age of 14 (19) and in this group there was a 5-year delay in establishing the diagnosis (19).

SLE can occur at any age, although it becomes more frequent after five years of age and is increasingly prevalent after the first decade of life (14). In retrospective reviews from France, Canada, and the United Kingdom, the median age of onset of juvenile-onset SLE was 12 to 13 years (134,135,137). Childhood SLE affects girls more often than boys (8:1), even in the prepubescent age group (4:1) (14).

The 2012 SLICC proposed revised SLE criteria are also used to classify children (138,139).

During my PhD, I performed a high-throughput study of the kidney miRNA signature of lupus nephritis in a pediatric cohort. This was particularly informative, considering that children with lupus nephritis have a more aggressive disease and co-morbidities, such as hypertension or diabetes mellitus, are less frequent. Pediatric cohorts are, therefore, essential to better understand the pathogenesis of this disease.

1.8 CLINICAL MANAGEMENT

When evaluating a patient with lupus, disease activity and disease severity should be determined. Disease activity refers to the reversible manifestations related to the underlying inflammatory process, while disease severity refers to the type and level of organ dysfunction. The degree of irreversible organ dysfunction is defined as damage. Features related to active SLE must be distinguished from chronic damage, drug toxicities or other comorbidities, including infection.

There is no consensus on what constitutes a disease flare, but most clinicians agree that a moderate or severe flare refers to a measurable increase in disease activity that is clinically meaningful enough to prompt a change in therapy (140,141).

In every clinical visit, disease activity and severity should be evaluated using clinical history, physical examination and specific tests. The physical examination should be thorough, including the complete observation of the skin, in order to identify a malar rash, discoid lesions or patchy alopecia. The clinician should also look for oral or nasopharyngeal ulcers and lymph nodes enlargement. The respiratory, cardiovascular, musculoskeletal and neurological systems should all be evaluated.

Regarding the laboratory tests required, it is recommended to check the complete blood count, erythrocyte sedimentation rate, C-reactive protein, serum creatinine, urinalysis with examination of the urinary sediment, spot urine protein and creatinine, titers of anti-dsDNA antibodies and complement levels (C3 and C4). Additional tests are required according with organ involvement.

The frequency of the clinical visits and of the laboratory tests is adjusted according to the patient's prior symptoms and current disease activity and severity. Even SLE patients with stable disease benefit from close follow-up at three- to four-month intervals (142). A study including over 500 SLE patients with mild or inactive disease found that one in four patients followed over a two-year period will have a laboratory abnormality without any clinical symptom that could lead to a change in management (143). The most useful laboratory tests to predict an SLE flare are an increase in the titer of anti-dsDNA antibodies and hypocomplementemia (144–147).

Several disease activity measures and indices have been developed as research tools. Examples of scoring systems for disease activity include the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (143), the Safety of Estrogens in Lupus Erythematosus: National Assessment-SLEDAI (SELENA-SLEDAI) (148), the Systemic Lupus Activity Measure (SLAM) (149), the British Isles Lupus Assessment Group (BILAG) (150), the European Consensus Lupus Activity Measurement (ECLAM) (151).

The Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/ACR-DI) is used to measure accumulated damage that has occurred since disease onset (152,153).

1.9 TREATMENT STRATEGIES

The treatment strategies used for the care of SLE patients are individualized according to the patients' preferences, clinical manifestations, disease activity and comorbidities. The main goals are to ensure long-term survival, prevent organ damage, minimize drug toxicity and improve quality of life.

As previously discussed, regular clinical monitoring is essential to assess disease activity, prevent and treat relapses, monitor the side effects of the drugs used and encourage adherence to treatment.

Since multiorgan system involvement is frequent, a multidisciplinary team is usually necessary to provide the best possible care for these patients.

It is also essential to educate patients on their role in disease management.

Several nonpharmacologic measures are important for disease control. Sun protection is one of the most important measures to prevent a disease flare. It is well known that exposure to ultraviolet (UV) light may exacerbate or induce the disease (154). It is, therefore, recommended that patients use UV-A and UV-B blocking sunscreens, with a sun protection factor ≥30. Medications that cause photosensitivity should also be avoided.

Interestingly, the majority of SLE patients have low serum levels of 25-hydroxycholecalciferol, probably due, at least in part, to the avoidance of sun exposure. Vitamin D levels should, therefore, be monitored periodically and patients with low levels of calcidiol should be treated with supplemental vitamin D (155).

Modifiable risk factors for coronary heart disease should always be addressed, including weight loss through dietary modification and exercise, use of statins and optimal blood pressure control. Smoking cessation should be recommended to all patients, since it increases the risk of active disease (156,157) and accelerated atherosclerosis (158,159). In patients with nephritis and/or hypertension, salt restriction should be advocated.

Notably, exercise is a very important tool to improve the global health of SLE patients and it has a great impact on the quality of life. Inactivity produced by the fatigue associated with disease activity or with acute illness causes a rapid loss of muscle mass,

bone demineralization and loss of stamina. Graded aerobic exercise is the best tool to improve fatigue in SLE patients (160).

Regarding immunizations, patients should receive appropriate immunizations prior to the institution of immunosuppressive therapies. Yearly influenza vaccine should be suggested. Live vaccines should not be administered to immunosuppressed patients, including those treated with prednisone at doses ≥ 20 mg/day for more than two weeks, azathioprine, micofenolate mofetil or biologic agents.

Concerning cutaneous lupus, the goal of treatment is to prevent long-term sequelae, namely telangiectasia, hyperpigmentation or hypopigmentation, alopecia, and scarring. For early superficial involvement, hydrocortisone may be a good option, but more potent topical corticosteroids with or without occlusion or intralesional corticosteroids should be used for thicker lesions. Other topical immunomodulatory agents, including tacrolimus and pimecrolimus, have been suggested to be effective for the treatment of cutaneous lupus (161–164).

Taking into account the clinical heterogeneity of SLE and the unpredictable course of the disease, the treatment strategy is highly individualized and is guided by the predominant symptoms, organ involvement and response to previous drugs.

Generally, patients with mild SLE manifestations may be treated with antimalarial drugs, and/or short-term use of low-dose glucocorticoids (≤ 7.5 mg prednisone/day). Patients with moderate lupus involvement, with significant but non-organ-threatening disease usually respond to hydroxychloroquine plus short-term therapy with 5 to 15 mg of prednisone/day. A steroid-sparing immunosuppressive agent is often required to control symptoms. Patients with major organ involvement generally require an initial period of intensive immunosuppressive therapy to control the disease, with high doses of systemic glucocorticoids (intravenous methylprednisolone 30mg/Kg for three days) used alone or in combination with other immunosuppressive agents, most frequently cyclophosphamide and mycophenolate mofetil. This induction phase is subsequently followed by a longer period of less intensive maintenance therapy to consolidate remission and prevent flares.

Independently of the disease manifestations, all patients with SLE should be treated with hydroxychloroquine (6.5 mg/kg per day to a maximum of 400 mg/day) or chloroquine (250 to 500mg/day) (165). Antimalarial agents provide benefit via a number

of mechanisms, including photoprotection, decrease of apoptosis, immunosuppression, and inhibition of antigen presentation, prostaglandin synthesis, lipid peroxidation, and proinflammatory cytokine synthesis (166). The benefits of antimalarial drugs in SLE are broad and include relief of constitutional symptoms and musculoskeletal and mucocutaneous manifestations. Antimalarial drugs are also the main drugs used to treat acute and subacute cutaneous lupus erythematosus and lupus panniculitis. There is also evidence that hydroxychloroquine reduces flares, thrombotic events, organ damage and mortality (165,167–170). In a nested case-control study it was shown that antimalarial drugs were associated with a 68% reduction in the risk of all thrombovascular events, after accounting for the effects of disease severity, disease duration and calendar year (169).

A six-month, randomized, double-blind, placebo-controlled study of the effect of discontinuing hydroxychloroquine sulfate treatment was performed in 47 SLE patients in remission (167). It was found that the relative risk of a flare was 2.5 times higher in the patients taking placebo than in those continuing to take hydroxychloroquine (16 of 22 patients vs. 9 of 25 had flares) and the time to a flare was shorter ($p = 0.02$) (167). The relative risk of a severe exacerbation of disease that required withdrawal from the study was 6.1 times higher for the patients taking placebo (5 of 22 patients vs. 1 of 25 had severe exacerbations of the disease) (167).

Toxicity related to antimalarial drugs is infrequent, mild and usually reversible (165). In conclusion, given the broad spectrum of beneficial effects and safety profile, hydroxychloroquine should be given to SLE patients during the course of the disease, irrespective of its activity or severity (165).

For patients with refractory or persistent arthritis, methotrexate might be an option. A systematic review of nine studies (three of which were randomized controlled trials) including SLE patients with predominantly mucocutaneous or arthritis features found that treatment with methotrexate was associated with a significant reduction in disease activity as well as in the average dose of glucocorticoids used (171). For patients with persistent arthritis unresponsive to methotrexate after three to six months of therapy, azathioprine might be a good option (172).

Azathioprine is a prodrug that is converted by thiopurine methyltransferase to mercaptopurine and methylnitroimidazole. The first interferes with the purine synthesis,

which affects both cellular and humoral immune functions. Azathioprine (2–3 mg/kg/day) is mainly used in SLE patients presenting with arthritis, mucocutaneous manifestations, and serositis, and as maintenance therapy in lupus nephritis (173). The side effects of this drug include a mild increased risk for infections, bone marrow suppression, hepatotoxicity and gastrointestinal intolerance. Patients with intermediate or low thiopurine methyltransferase activity have an increased risk of myelosuppression. It is, therefore, recommended genotyping or phenotyping the thiopurine methyltransferase before starting azathioprine (174).

Cyclophosphamide is an alkylating agent that interferes with DNA replication by forming DNA crosslinks. In a 1986, NIH landmark study of lupus nephritis treatment, it was shown that, after 10 years, renal function was better preserved with combination therapy of prednisone and intravenous pulse cyclophosphamide than prednisone alone (175). Cyclophosphamide is associated, however, with important side effects, including major infections, infertility, bladder toxicity, and increased risk of malignancy (176). Some of these toxicities are dose-dependent (176). In a study of 216 women with SLE who had been treated with cyclophosphamide, levels of the anti-müllerian hormone, a surrogate of ovarian reserve, were inversely correlated with the cumulative dose of cyclophosphamide administered (177).

For the induction therapy of lupus nephritis, American, European and Asian guidelines recommend a monthly course of intravenous pulse cyclophosphamide for six months (0.5 g/m^2 to a maximum of 1.5 g) (178–180). A lower-dose cyclophosphamide regimen has been used in Europe (the Euro-Lupus regimen) composed by six biweekly low-dose pulses of cyclophosphamide (500 mg each) (181). The long-term efficacy of the Euro-Lupus regimen has been proved in a limited number of Caucasian patients with mild renal disease (181). The efficacy of this regimen needs to be further addressed in other ethnical groups and in patients with more serious forms of lupus nephritis (176).

Currently, cyclophosphamide is not the first-line option for the treatment of lupus nephritis in the majority of the centers, considering its side effects. Nevertheless, cyclophosphamide still plays an important role in the management of patients with lupus nephritis with impaired or rapidly deteriorating renal function, as salvage therapy for recalcitrant disease and in patients with neuropsychiatric involvement.

Mycophenolate mofetil is a prodrug of mycophenolic acid, which inhibits the

enzyme inosine monophosphate dehydrogenase, an essential step in the *de novo* synthesis of guanosine nucleotides. The latter are required for DNA formation and, consequently, lymphocyte proliferation. As lymphocytes are highly dependent on this pathway for DNA synthesis, mycophenolate mofetil is considered to be a more lymphocyte-selective immunosuppressive agent than other purine antagonists, such as azathioprine. The main adverse effects of mycophenolate mofetil are gastrointestinal intolerance, bone marrow suppression, and increased risk of infections.

In recent years, mycophenolate mofetil has emerged as the first-line option for induction therapy of lupus nephritis (182,183). A pilot study showed that mycophenolate mofetil (2 g/day) was equally effective at 12 months as sequential oral cyclophosphamide and azathioprine (184). Furthermore, two large randomized controlled trials demonstrated, at six months, the non-inferiority of mycophenolate mofetil (3g/day) to the NIH intravenous pulse cyclophosphamide for the treatment of lupus nephritis (182,183). Furthermore, mycophenolate mofetil was found to be more effective than cyclophosphamide in Hispanics and African Americans (response rate 60% versus 39%; P=0.03) (183). In conclusion, considering the equivalent efficacy of mycophenolate mofetil to cyclophosphamide, with reduced gonadal toxicity and oncogenicity, mycophenolate mofetil should be considered as first-line induction treatment of lupus nephritis. This is particularly important in women who wish to have children and in Hispanics and African Americans, since it is much better tolerated and more effective than cyclophosphamide in this group.

Mycophenolate mofetil has also been used as maintenance therapy for lupus nephritis. In the ALMS study (N = 227) mycophenolate mofetil (2 g/day) was shown to be superior to azathioprine in the prevention of renal flares, after 3 years, in those who responded to induction therapy with either intravenous pulse cyclophosphamide or mycophenolate mofetil (185). On the other hand, recently, a randomized controlled study of Caucasian patients with proliferative lupus nephritis, who received the Euro-Lupus cyclophosphamide regimen as induction (N = 105), failed to show that mycophenolate mofetil was superior to azathioprine for lupus nephritis maintenance at 10 years (186). Further studies are thus needed to address the real value of mycophenolate mofetil and azathioprine in the maintenance therapy for lupus nephritis.

Combination of immunosuppressive agents in order to obtain a synergistic effect

has long been used in organ transplantation to reduce the risk of rejection and is now also being tested for the treatment of lupus nephritis (176). A 24-week randomized, open-label, multicenter study analyzed the effect of combining mycophenolate mofetil (1g/day) and tacrolimus (4mg/day) versus intravenous cyclophosphamide (0.5 to 1.0 g/m^2), every 4 weeks, for six months (187). After 24 weeks of therapy more patients in the multitarget group (46%) than in the cyclophosphamide group (26%) showed complete remission ($P<0.0001$) (187).

Tacrolimus is a calcineurin inhibitor that suppresses the transcription of interleukin (IL) 2 and, consequently, inhibits T cell activation. B cell activation, class-switching and immunoglobulin production are, as a result, compromised. Tacrolimus is also thought to reduce proteinuria through the stabilization of the actin cytoskeleton in podocytes (188). In a recent randomized controlled trial of 150 patients with biopsy-proven active lupus nephritis (Class III/IV/V), tacrolimus (0.06–0.1 mg/kg/day) was shown to be non-inferior to mycophenolate mofetil (2–3 g/day) as induction therapy in terms of the complete renal response rate at 6 months (189). The long-term efficacy of tacrolimus for the treatment of lupus nephritis is not yet available.

Belimumab is a human monoclonal antibody that inhibits BLyS (B Lymphocyte Stimulator), also known as BAFF (B Cell Activating Factor). BLyS is elevated in SLE patients, and it may play a role in the pathogenesis of the disease by promoting the formation and survival of memory B cells and plasmablasts. A randomized, placebo-controlled, phase 3 trial of 867 SLE patients showed that belimumab reduced disease activity (190). This drug is usually suggested to patients with active musculoskeletal or cutaneous disease that are unresponsive to standard therapy. It is not recommended, however, to treat lupus nephritis or central nervous system involvement, since there is still no evidence that it is helpful in these cases (190).

Rituximab, the B-cell-depleting chimeric monoclonal antibody anti-CD 20, has also been used to treat SLE patients (191). A systematic review of 188 patients treated with rituximab identified 171 (91%) patients with a significant improvement in one or more of the systemic SLE manifestations (192). There were 103 patients with lupus nephritis, with an overall rate of therapeutic response of 91% (192). Adverse events were reported in 44 (23%) patients, mainly infections (19%) (192). An analysis, in 2010, of the French AutoImmunity and Rituximab Registry also showed a satisfactory tolerance profile and

clinical efficacy (193). A systematic review, performed in 2014, included 1,231 patients and clearly showed that rituximab decreased disease activity, improved arthritis and thrombocytopenia, increased complement, decreased anti-dsDNA antibodies and had a steroid-sparing effect (194). Recent studies, however, have shown some disappointing results. The EXPLORER trial was a randomized, double-blind, phase II/III study that enrolled patients with moderately-to-severely active SLE (195). In this study no differences were noted between placebo and rituximab (195). In another randomized trial 144 patients received rituximab or placebo (196). Although, rituximab led to greater reductions in anti-dsDNA antibodies and increased C3 and C4 levels, it did not improve clinical outcomes after 1 year of treatment (196). Some of the negative results obtained in clinical trials have been attributed to methodologic problems and also to the heterogeneity of the disease, which makes it difficult to show differences between treatment interventions and placebo. Despite the lack of success of the clinical trials reported so far, rituximab continues to be a valuable off label drug, particularly important for the treatment of refractory hematologic and renal disease.

Concerning the treatment of neurolupus, clinical trials are scarce and most of the data are extracted from case series and case reports. One of the few randomized control trials studied 32 patients with SLE and an acute onset of severe neurologic manifestations (seizures, optic neuritis, neuropathy, coma, brainstem disease, or transverse myelitis). It compared treatment with methylprednisolone and cyclophosphamide. The great majority (18/19) of the patients receiving cyclophosphamide and 7 of the 13 receiving methylprednisolone had at least a 20% improvement in a pertinent neurologic measure (197). Response to cyclophosphamide was also assessed in another study of 31 patients with severe neuropsychiatric lupus, 90% of whom had failed previous treatment strategies. Eight patients received plasmapheresis in addition to cyclophosphamide. Substantial improvement was seen in 61% and partial improvement was seen in 29% (198). Currently, the recommended drugs for the control of acute neurologic symptoms are high-dose glucocorticoids (prednisolone 1mg/Kg/day, with a tapering schedule of 3-6 months with or without a previous three days of intravenous methylprednisolone 500-1000 mg/day) and intravenous cyclophosphamide (500-750mg/m^2/month) (199). After six months of treatment, cyclophosphamide can be continued in the same dose every three months for

18 months, or other immunosuppressive therapy can be considered for maintenance, including azathioprine and mycophenolate mofetil. Rituximab, intravenous immunoglobulins, or plasmapheresis may be used if response is not achieved (200).

The recommendations for primary and secondary cerebrovascular disease prevention in SLE patients are still under debate. The European League Against Rheumatism (EULAR) guidelines suggest anticoagulation for secondary prevention of arterial events in SLE patients with persistently positive moderate-to-high titers of antiphospholipids antibodies (199). Some authors suggest a target INR of 2.0-3.0 in patients with antiphospholipid syndrome after a first venous event and >3.0 for those with recurrent and/or arterial events (201). There is controversy, however, regarding the INR target. Two randomized control trials of 114 and 109 patients with primary and SLE-related anti-phospholipid syndrome have showed no superiority of high-intensity warfarin (target INR 3.0–4.0) over moderate-intensity warfarin (target INR 2.0–3.0) for secondary prophylaxis (202,203). New studies are mandatory to better understand which is the best approach for this group of patients.

Finally, hematopoietic stem cell transplantation has been used for the treatment of severe autoimmune diseases, including SLE. It is recommended a nonmyeloablative immunosuppressive conditioning regime, usually high dose cyclophosphamide and another agent, such as anti-thymocyte globulin. Autologous rather than allogeneic hematopoietic stem cell transplantation has generally been preferred, because of the morbidity and mortality associated with graft-versus-host disease.

Hematopoietic stem cell transplantation was examined in a phase I study, in which seven patients with severe SLE received high-dose chemotherapy followed by autologous stem cell transplantation (204). These patients had active diffuse proliferative glomerulonephritis, cerebritis, myelitis, and/or vasculitis despite six cycles of cyclophosphamide (204). At a median follow-up of 25 months, all patients were free from signs of active lupus and renal, cardiac, and pulmonary function and serologic markers had improved despite either no immunosuppression or small residual doses of glucocorticoids. It is hypothesized that stem cell transplantation is beneficial in this setting, since it provides a period without memory T cell influence, during which the maturation of new lymphocyte progenitors can occur without recruitment to anti-self activity (204). Hematopoietic stem cell transplantation is associated with significant

morbidity and mortality, thus the precise indications for this treatment should be carefully addressed.

High-dose cyclophosphamide (50mg/Kg/day for four consecutive days) without hematopoietic stem cell rescue has also been used in a variety of severe autoimmune diseases, including SLE (205). Because high-dose cyclophosphamide spares hematopoietic stem cells, it can be safely administered without stem cell support. In one study 90% (36/40) of SLE patients had a partial or complete response to treatment. The median duration of response was 12 months (range 3-48 months) (205). The median of neutropenia (absolute neutrophil count below 0.5 x 10^9/L) duration was 9 days, similar to what is seen after autologous stem cell rescue (205). Febrile neutropenia occurred in less than 50% of patients and the median number of days in the hospital was six (205). No treatment-related deaths were observed, while the reported 100-day transplant-related mortality was 11% in patients with SLE (205). High-dose cyclophosphamide is not superior to monthly pulse dose cyclophosphamide and it is not recommended as first line therapy for lupus nephritis. Nevertheless, high-dose cyclophosphamide can be effective salvage therapy for patients with refractory SLE, especially those with neurologic manifestations.

In conclusion, nowadays we have a diverse group of drugs that act upon the imune system and control SLE manifestations. Nevertheless, these drugs are not capable of transforming SLE into a curable disease and are responsible for serious side effects. New tailored therapies are, therefore, much-needed. Once again, only the study of the pathogenesis of the disease may help us to find new ways to improve the care of these patients.

1.10 PROGNOSIS

Overall improvements in medical care including the well-judged use of immunosuppressive drugs and the availability of antibiotics, antihypertensive drugs and renal replacement therapy have led to increased survival of SLE patients in the past decades (1). The 5-year survival rate among 99 patients followed at Johns Hopkins University from 1949 to 1953 was 50% (206). Since the mid-1970s, it has been reported in the United States, Canada and Latin America, a 5-year survival rate over 90% and 20

year survival rates of around 80% (4,5,9,12,52,57,207–218). Although the improvement in survival in SLE patients has been greater than that observed in general population, life expectancy in this group is still below the one reported to other comparable demographic groups (1). SLE patients' mortality rates are, indeed, 2 to 5 times higher than that of the general population (219,220). Cause of death varies depending on disease duration (219). The major causes of death in the first years of illness are active disease and infection due to immunosuppression, while causes of late death include mainly cardiovascular disease (19,221,222).

Long-term morbidity is also a major issue in SLE, since it affects the ability to work, being associated with a significant burden of direct and indirect costs (1). Fatigue, cognitive dysfunction, glucocorticoid-induced avascular necrosis of the hips and knees and chronic pain are common problems, which have an enormous impact on the quality of life.

Ethnicity has been reported as an important prognostic factor. As previously discussed, Hispanic, African American and Asian SLE patients tend to have more hematological, serosal, neurological and renal manifestations than Caucasian patients (54,213,216,223,224). These groups also accumulate more damage over time (213) and at a faster pace (225). Higher mortality risks have been reported in African American, Hispanic and Native American groups. Notably, this differential risk disappears if the data are adjusted for socioeconomic status, namely income, insurance status or educational level. This is an important element to take into account when analyzing the role of ethnicity in mortality (1). Some of these factors are certainly amenable to interventions that may have a direct impact on the mortality of these patients.

In a large international SLE cohort (23 centers; 9,547 patients) not only ethnicity was found to be an important prognostic factor, but also age at diagnosis (226). Other poor prognostic factors for survival in SLE also include high disease activity at diagnosis, nephritis, neuropsychiatric involvement, thrombocytopenia, lung involvement, hypertension and presence of antiphospholipid antibodies (220,227).

Despite the considerable improvements in the care of SLE patients, the burden of the disease is still high and there are innumerous clinical challenges. Only the combined effort of physicians and scientists in the field will allow to better understand this disease, which hopefully will translate in clinical advances for these patients.

CHAPTER 2

Pathogenesis of Systemic Lupus Erythematosus

2.1 OVERVIEW

The break of self-tolerance is classically considered the central phenomenon in SLE pathogenesis. Several mechanisms may contribute to this central event, including the imbalance between the production and the disposal of apoptotic material. Usually nuclear antigens are not accessible to the immune system. During the course of apoptosis, however, blebs appear that contain fragmented cellular material including nuclear antigens (228). In SLE there is an increase in apoptosis, which is further intensified by UV light, infections and toxins, which are well-known SLE triggers. In addition, SLE patients have defective mechanisms of apoptotic waste removal, which globally cause the accumulation of apoptotic debris (229). The persistence of exposed nuclear autoantigens contributes to the break of self-tolerance, since these autoantigens are then presented by dendritic cells to stochastically generated autoreactive B cells, in germinal centers of secondary lymphoid organs. This results in the loss of self-tolerance and production of autoantibodies and immune complexes, a unifying feature of SLE (229). Once activated, B cells can mature, expand and begin to secrete more antibodies, thus enhancing the response. The autoantibodies identified in SLE are high affinity, somatically mutated and IgG, which suggest that they have arisen in the germinal center, where T cells and B cells interact to promote class switching. The immune complexes activate complement and induce inflammation, causing tissue damage. Moreover, plasmacytoid dendritic cells produce high amounts of interferon (IFN) α upon immune complexes uptake, thereby contributing to the characteristic IFNα signature described in SLE.

Another mechanism responsible for inflammation in SLE is the direct activation of nucleic acid recognition receptors by the apoptotic debris containing nucleic acids (230). Once again the activation of these pathways is strongly associated with IFNα production. These and other cytokines promote B cell differentiation and loss of tolerance, enhancing the overall inflammatory effect. SLE etiopathogenesis is, therefore, a vicious cycle of autoantigen exposure, autoantibody production, chronic inflammation and damage. Other cytokines also participate in this amplification loop, including BAFF, IL6, IL10, IL17 and IL23. IL2 is decreased in SLE and contributes to the imbalance between Th17 cells and regulatory T cells.

This is still an imperfect perception of the pathogenesis of SLE and there are certainly many unanswered questions regarding this subject. One of the most pressing topics is unveiling the phenomena responsible for the phenotypic heterogeneity in SLE.

In this chapter we will analyze the currently known mechanisms responsible for SLE pathogenesis, as well as the recent insights that extend this model and offer the potential for novel interventions.

2.2 MECHANISMS OF DISEASE IN SLE

2.2.1 Apoptosis

Changes in cell death pathways, including apoptosis and the neutrophil-specific kind of cell death called NETosis are a source of autoantigens in SLE (229,230).

Apoptosis is a normal process of tissue homeostasis, characterized by specific morphological features, including pyknosis, karyorrhexis, cytoskeleton remodeling and plasma membrane blebbing (231). Apoptosis is usually an immunologically silent process, because apoptotic cells are immediately phagocytosed and degraded. This clearance process starts when the apoptotic cell secretes mediators, including ATP, UTP, sphingosine-1-phosphate, lysophosphatidylcholine, and fractalkine (CX3CL1), which stimulate migration and activation of leukocytes (229). The apoptotic cell also exposes the phospholipid phosphatidylserine on the outer leaflet of the plasma membrane, which enhances recognition and engulfment by the phagocytes. The uptake of apoptotic cells is followed by the secretion of anti-inflammatory cytokines, including IL10. The latter inhibits the further recruitment of macrophages to the site (232,233).

In SLE the clearance of apoptosis debris is impaired. Macrophages from SLE patients have reduced adherence, which may be mediated, at least in part, by a reduction on the expression of the cell adhesion receptor CD 44 (234). Furthermore, *in vitro*-differentiated macrophages from SLE patients have a decreased and delayed engulfment capacity for autologous apoptotic material (229,232,235).

Dead cell clearance not only depends upon functioning phagocytes, but also upon soluble proteins, which function as opsonins and bridging molecules. C-reactive protein, serum amyloid P component, C1q, IgM, and mannose binding lectin are a few of

these molecules. Globally they constitute a redundant back-up mechanism.

C1q was found to be essential for effective uptake of degraded chromatin by phagocytes (235). In SLE there is usually a complement deficiency, which may be primary, due to genetic defects on the complement pathway, or secondary, due to consumption as the disease progresses. The decrease on available complement molecules may lead to inefficient clearance of apoptotic cells (232). Moreover, complement components are involved in the clearance of immune complexes. It has been shown, for instance, that C4-deficient mice had a lupus like phenotype, characterized by delay in clearance of circulating immune complexes, glomerular deposition of immune complexes, glomerulonephritis and splenomegaly (236). The defective clearance of immune complexes in SLE can, therefore, contribute to its accumulation and may further lead to inflammatory damage in organs, such as kidneys.

C-reactive protein, serum amyloid P component, and PTX3 also bind apoptotic material and activate the classical pathway of complement. This increases phagocytosis of dead and dying cells with a subsequent anti-inflammatory response. Interestingly, a *CRP* polymorphism associated with low levels of C-reactive protein was associated with SLE development (237). Moreover, it has been demonstrated that IFNα has an inhibitory effect on the secretion of C-reactive protein, which explains why the levels of C-reactive protein are not increased during SLE flares (238).

Finally, DNase I participates in the clearance of nuclear debris, since it is responsible for chromatin degradation (239,240). Interestingly, DNAse I deficiency is known to contribute to SLE pathogenesis (241).

In conclusion, these data globally support the hypothesis that defective disposal of potentially immunogenic material is a contributory factor in SLE pathogenesis.

2.2.2 NETosis

NETosis is a mechanism of neutrophil cell death. Following an activation signal, NADPH oxidase and superoxide dismutase generated H_2O_2 promotes the migration of elastase to the nucleus. This enzyme cleaves histones and elicits chromatin decondensation. In addition, the enzyme peptylarginine deiminase 4 (PAD4) changes the positively charged arginine residues of histones to more neutrally charged citrulline residues, which dampens the electrostatic interactions between histones and negatively

charged DNA. The nuclear and granular membranes degrade and finally the chromatin/gAMP meshwork is extruded from the cell (242). This material is designated as NETs, since it contains a chromatin fiber web, as well as bactericidal, immunostimulatory proteins, and autoantigens.

NETosis has multiple pro-inflammatory consequences, including stimulating plasmacytoid dendritic cells to produce IFNα (243). NETs contain DNA as well as LL37 and HMGB1, neutrophil proteins that facilitate the uptake and recognition of DNA in plasmacytoid dendritic cells (244). NETs activate these cells to produce high levels of IFNα in a toll-like-receptor (TLR) 9 dependent manner, contributing, therefore, to the type I IFN signature of SLE (244,245). In turn IFNα primes neutrophils for NET release, suggesting a positive feedback loop between NETosis and IFN signaling (244).

NETs also activate the NLRP3 inflammasome in macrophages leading to enhanced IL1β and IL18 production (246). The latter is able to stimulate NETosis, once again showing the existence of a feed-forward inflammatory loop (246).

In SLE there is a population of low density granulocytes, which have a propensity to form NETs, externalizing enhanced levels of autoantigens and immunostimulatory molecules (229). Interestingly, NGAL is a granular protein externalized in NETs and is a lupus nephritis biomarker, correlating with disease activity (247,248).

SLE patients were found to develop autoantibodies to both the self-DNA and antimicrobial peptides in NETs, indicating that NETs could serve as autoantigens to trigger B cell activation (245). In addition, the skin and the kidneys of SLE patients were shown to have infiltration by netting neutrophils and there was an association between tissue NETosis and the levels of anti-dsDNA antibodies (243).

The NETosis prone low-density granulocytes also externalize enhanced levels of metalloproteinase 9 (MMP 9), which activates MMP2 in endothelial cells (249). This pathway, in a mouse model, specifically impairs endothelium-dependent vasorelaxation and induces endothelial cell apoptosis (249), contributing, therefore, to endothelial cell dysfunction and atherosclerosis. Moreover, SLE patients show higher levels of immune complexes containing MMP-9 and anti-MMP-9 antibodies that may potentiate this process by enhancing NET formation (249).

NETs can also contribute to atherosclerosis by other mechanisms. Externalized material contains hyperacetylated histones, which activate macrophages (250), and

active oxidative enzymes, which can modify the high-density lipoprotein, rendering the lipoprotein proatherogenic (251).

In analogy to the deficient clearance of apoptotic cells, the inefficient degradation of NETs can also be involved in the generation of autoantibodies (252). NETs are not efficiently degraded by sera from SLE patients, while healthy donor serum efficiently causes NET degradation (253). One possible explanation for this observation is the existence of anti-DNase antibodies in SLE patients. Notably, the majority of SLE patients are positive for anti-DNase antibodies (254). These recognize a conserved epitope near the catalytic site of DNase and, therefore, protect NETs from degradation (254). Other possible mechanism is the presence of DNase inhibitors (253). NET-bound autoantibodies cause C1q deposition, which directly inhibits DNase I and prevents NET degradation, exacerbating the exposure of auto-antigens (252).

Finally, the role of NETs in SLE pathogenesis was also highlighted by the effect of PAD inhibition *in vivo* (242). PAD4 is required for NET formation and when it was inhibited in lupus-prone mouse models, there was significant improvement of skin, kidney and vascular disease (255,256). PAD inhibition in atherosclerosis-prone mouse models also decreased plaque formation, showing that NET inhibition can have a vasculoprotective effect (257).

In conclusion, recent evidence suggests that NETs, like apoptotic debris, may serve as a source of autoantigens and as stimulus to inflammation, participating in SLE pathogenesis. The study of NETs is, therefore, an emerging aspect of SLE research, and NETs might represent a novel diagnostic and therapeutic target.

2.2.3 Nucleic acid sensors

The accumulated apoptotic and NETosis debris can trigger TLRs and nucleic acid sensors. TLRs are pattern recognition receptors that are involved in the innate host defense against pathogens. In addition, TLRs also recognize nucleic acids, released when tissue damage or apoptosis occur (258).

Immune cells, including B cells, some T cells, dendritic cells and macrophages, as well as non-immune cells, such as epithelial cells and fibroblasts, express TLRs. These are trans-membrane glycoproteins, which have an extracellular N-terminal domain and an intracellular C-terminal region. The former recognizes the molecular patterns and the

latter plays a critical role in the activation of the downstream signaling through the interaction and recruitment of several adaptor molecules, including MyD88, TIRAP, TRIF and TRAM.

TLRs have different locations within the cells and their expression changes greatly with the cytokine milieu. TLR2, TLR4, TLR5 and TLR6 are located at the cell membrane, while TLR3, TLR7, TLR8 and TLR9 have an intracellular location.

Mouse and human studies consistently suggest that the intracellular TLRs contribute to SLE pathogenesis. In pristane-treated mice, for instance, TLR7, a receptor for single-stranded RNA, was specifically required for the production of RNA-reactive autoantibodies and for the development of glomerulonephritis (259). Moreover, in MRL lpr mice, a TLR7 antagonist prevented kidney injury (260). Pharmacologic activation of TLR7 in MRL lpr/lpr mice induced IFNα release, renal immune complex deposition and renal tissue injury (261). In addition, a *Tlr7* translocation, which caused *Tlr7* overexpression, accelerated systemic autoimmunity in a lupus mouse model (262). Whereas a modest increase in *Tlr7* gene dosage promoted autoreactive lymphocytes with RNA specificities and myeloid cell proliferation, a substantial increase in *Tlr7* expression caused a dramatic dendritic cell dysregulation and fatal acute inflammatory injury (263).

TREML4 is an essential positive regulator of TLR7 signaling. Macrophages from *Treml4*(-/-) mice were found to be hyporesponsive to TLR7 agonists and failed to produce type I IFNs, due, in part, to decreased recruitment of the adaptor MyD88 to TLR7 (264). Moreover, in MRL/lpr mice TREML4 deficiency reduced the production of inflammatory cytokines and autoantibodies (265).

TLR9 is a receptor for DNA containing unmethylated CpG sequence motifs. It was found that SLE patients with active disease had an increased number of TLR9-expressing B cells and monocytes when compared to patients with inactive disease (266). Moreover, the number of TLR9 expressing-B cells correlated with anti-dsDNA antibodies levels (266).

It was also found that in TLR9-deficient lupus-prone mice the generation of anti-dsDNA and antichromatin autoantibodies was specifically inhibited, while other autoantibodies levels, such as anti-Sm, were maintained or even increased (267). Notably, TLR9 deficiency exacerbated disease activity in MRL/lpr lupus-prone mice (268), whereas treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 caused

reduction of autoantibody production and improvement of disease symptoms (269). This apparent paradox can be explained, because, upon activation, TLR7 and TLR9 are transported from the endoplasmic reticulum to endolysosomes by the protein UNC93B1 (270). TLR9 competes with TLR7 for UNC93B1-dependent trafficking and predominates over TLR7 (270). TLR9 depletion increases TLR7 trafficking and signaling, resulting in systemic autoimmunity (270). In conclusion, the role of TLR9 in SLE is complex, since it drives DNA autoantibody production and suppresses TLR7 signaling.

In addition to TLR9, TLR 8, a receptor for single-stranded RNA, also suppresses TLR7-mediated systemic autoimmunity. *Tlr8*(-/-) mice had increased serum levels of autoantibodies and glomerulonephritis, a phenotype not seen in *Tlr8*(-/-)*Tlr7*(-/-) mice (271). In C57BL/6 mice it was also confirmed that TLR8 on dendritic cells and TLR9 on B cells restrain TLR7-mediated spontaneous autoimmunity (272). Accordingly, in MRL/lpr mice, B-cell-specific deletion of MyD88, a TLR7/TLR9 adaptor, decreased dramatically autoantibody production, class switching and T-cell activation. Notably, only MyD88 deficiency in B cells, but not in dendritic cells, abrogated lupus nephritis (273).

TLR3, another intracellular TLR, which is a receptor for double-stranded RNA, is also associated with SLE pathogenesis. In MRL/lpr mice, for instance, TLR3 activation induced lupus nephritis (274). TLR3 expression was also found to be up-regulated in leukocytes from SLE patients (275).

In addition to the TLR signaling pathways, recent studies have demonstrated the existence of a cytosolic TLR-independent system for the recognition of acid nucleics. RIG1 and MDA5 recognize double-stranded RNA and activate the IPS1 dependent cytosolic signaling pathway, leading to the synthesis of type I IFNs and inflammatory proteins.

Overall, the main result of activation of these pathways and TLRs is type I IFN production. Important insights have come from the study of rare monogenic disorders with a lupus-like phenotype – the type I IFNopathies. These comprise a set of monogenic diseases linked by overproduction of type I IFN (276). One of the main phenotypic categories is that of Aicardi-Goutieres syndrome. The most frequent manifestations of this syndrome are an early onset progressive inflammatory brain disease, chilblains and glaucoma. Mutations in *TREX1*, *RNASEH2A/B/C*, *SAMHD1*, *ADAR*, or *IFIH1* are associated with this phenotype and all these gene defects drive overproduction of type I IFN (276). TREX1, for instance, degrades single-stranded and double-stranded DNA. TREX1

deficiency may, therefore, trigger autoimmunity through the accumulation of DNA species (276). These are sensed by cyclic GMP-AMP (cGAMP) synthase, which produce cGAMP. This is a ligand for stimulator of IFN genes protein (STING), which leads to the production of type I IFN via activation of TBK1–IRF3 and possibly NF-κB (276). This syndrome is thus an argument in favor of the importance of the clearance of nuclear debris to avoid the break of tolerance and autoimmunity. The monogenic disorders associated with SLE are further discussed in Chapter 3.

2.2.4 Soluble mediators

Patients with SLE have a significantly altered cytokine profile, which reflects the underlying immune cell dysfunction and further contributes to the pathogenesis of the disease (277).

As previously discussed, one of the hallmarks of SLE is the IFNα signature. SLE patients have higher serum levels of IFNα (278) and an overexpression of IFNα-induced genes in peripheral blood immune cells (279–282). In addition, transcripts of IFNα and IFNα-induced genes have been detected in inflamed kidney and skin from SLE patients (283,284). Genetic studies further link the type I IFN to SLE, because specific haplotypes of IRF5 and IRF7 confer increased expression of IFNα as well as increased risk for SLE (285–287). Epigenetic studies also reveal that type I IFN-induced genes are hypomethylated in SLE patients (288). Finally, a direct pathogenic role for IFNα in lupus is also supported by mouse models, in which exogenous administration of IFNα exacerbates disease (289–292).

Different cells can produce small amounts of type I IFN, but the plasmacytoid dendritic cell is the main cell type responsible for the production of this cytokine. As described in the previous sections of this chapter, IFNα production is induced by viruses, bacteria or other microbes that stimulate pattern recognition receptors, including endosomal TLRs, the cytoplasmic RIG1 receptors and the intracellular dsDNA sensors DAI and cGAMP synthetase (293). Nucleic acids from endocytosed viruses stimulate the endosomal TLR7 and TLR9, which activate the MyD88-signalling pathway that ultimately leads to the phosphorylation of IRF3, IRF5 and IRF7 and the transcription of type I IFN mRNA (293). Infections can, therefore, increase the levels of type I IFN and,

consequently, contribute to an SLE flare. Nevertheless, the high levels of this cytokine seen in SLE are caused not by infections, but by endogenous inducers, namely nucleic acid-containing immune complexes (293), which are internalized via FcγRIIa and activate TLR7 and TLR9 in plasmacytoid dendritic cells (294). Such interferogenic immune complexes can be created *in vitro* by combining purified IgG from SLE patients and apoptotic cell material (295). This is another factor linking the defective clearance of apoptosis debris with SLE pathogenesis.

Another type of endogenous IFN inducers that stimulate plasmacytoid dendritic cells is self-nucleic acids in complex with RNA- or DNA-binding protein, such as HMGB1 and LL37 (245,296), which are present in NETs.

Regarding cell–cell interactions, natural killer cells, B cells and platelets promote IFNα production by plasmacytoid dendritic cells (293,297–299).

Interestingly, the production of type I IFNs in plasmacytoid cells is increased by estradiol *in vivo* through cell-intrinsic estrogen receptor α signaling (300). This may contribute, at least in part, to the differences in SLE prevalence between the genders.

Regarding the negative regulators, monocytes inhibit the increase of IFNα through the secretion of TNF, prostaglandin E2 and reactive oxygen species. This regulatory pathway is defective in SLE, since, as previously mentioned, reactive oxygen species generation is impaired (293). C1q is another negative regulator, since there is a preferential uptake of C1q-containing immune complexes by monocytes instead of plasmacytoid dendritic cells, thereby decreasing type I IFN production. This mechanism may explain the association between C1q deficiency and SLE (293).

Considering the effects of IFNα, it binds the ubiquitously expressed heterodimeric IFN α/β receptor (IFNAR), activating JAK1 and TYK2 and forming a transcription factor complex consisting of STAT1, STAT2 and IRF9. This transcription factor complex binds to IFN-stimulated response elements in promoters of IFN-regulated genes and initiates transcription (293).

In addition to a direct antiviral effect, IFNα has multiple pro-inflammatory functions. With regard to the innate immune system, type I IFN enhances Natural Killer cell cytotoxicity and IFNγ secretion, promotes the maturation of monocytes to antigen-presenting cells and induces the expression of MHC molecules and co-stimulatory molecules on dendritic cells (293). Type I IFN also have direct effects on T cells by

suppressing the differentiation of T regulatory cells (293). Furthermore, a persistent type I IFN stimulation promotes the development of T follicular helper cells, which support B cell activation (301). B cells are also directly activated by type I IFN, generating non-Ig-secreting plasma blasts, which further differentiate into Ig-secreting plasma cells when exposed to IL6 (302). Finally, type I IFN exerts a positive feedback regulation on the IFNα production through the upregulation of TLR7 and IRF7 expression in plasmacytoid dendritic cells (293).

All these findings suggest a central role for IFNα in the pathogenesis of SLE and have prompted the study of drugs targeting IFNα for SLE treatment. In fact, one of the most commonly used drugs for SLE treatment, hydroxychloroquine, downregulates the type I IFN signature (293). Hydroxychloroquine blocks the binding of TLR7/9 to nucleic acids (303) and inhibits the DNA sensor cGMP synthase (304).

The effect of anti-IFNα monoclonal antibodies has been evaluated in several clinical trials (305–308), as well as the effect of antibodies targeting the IFN α/β receptor. The anti-IFNα monoclonal antibodies do not completely neutralize the IFNα signature, possibly because they do not block all the IFNα and type I IFN subtypes. The inhibition of the IFNα receptor with anifrolumab seems, therefore, more promising.

BAFF is another cytokine particularly important in SLE, since belimumab, an anti-BAFF monoclonal antibody, was the first drug to be approved for the treatment of SLE in several decades, as was described in Chapter 1.

BAFF is a critical factor for B cell homeostasis. The high levels of BAFF detected in the serum of SLE patients promote autoantibody production (309). In a mouse model, overexpression of BAFF promoted anti-dsDNA B cell maturation and secretion of antibodies, suggesting that BAFF rescues a subset of anti-dsDNA B cells from a regulatory checkpoint in the transitional stage of development (310). A short course of selective blockade of BAFF alone was sufficient to prevent and treat nephritis in NZM2410 mice despite the formation of pathogenic autoantibodies (311). In humans prolonged BAFF inhibition decreases the survival of newly formed B cells, while not having the same effect on memory B cells and plasma cells (312). It is hypothesized, therefore, that the high BAFF levels seen in SLE may reduce the stringency of B cell selection, allowing autoreactive clones to persist in the periphery. Notably, B cell depletion therapy in SLE is

followed by an increase in BAFF levels, raising concern that the repopulating B cells may be more autoreactive in nature.

The levels of other cytokines are also altered in SLE, including IL2. The expression of this cytokine is regulated mainly at the transcriptional level by the binding of transcription factors to the IL2 promoter/enhancer region (313). In SLE there is a higher expression of CREMα, which binds to the IL2 promoter in T cells and represses the transcription of the IL2 gene (314). Treatment of normal T cells with SLE serum resulted in increased expression of CREM and decreased IL2 production (314). The effect of SLE serum resided within the IgG fraction and was specifically attributed to anti-TCR/CD3 autoantibodies (314).

Il2 plays a role as an auto- and paracrine growth factor during the first 48 to 72 h of T cell activation (315). Paradoxically, its absence has been linked to development of lethal autoimmunity in mice (315). This apparent contradiction has been partially explained by the fact that IL2 is an essential cytokine to maintain the balance between Th17 and regulatory T cells (315). IL2 promotes activation-induced cell death of lymphocytes, generates regulatory T cells and, signaling via STAT5, constrains Th17 cell generation (316). Recently, it was shown in a lupus prone mouse model that administration of low dose IL2 increased regulatory T cell numbers and function and it suppressed IL17 production and kidney damage (317).

Globally, the data show that the limited IL2 levels seen in SLE skew the balance between regulatory T cells and Th17 in favor of the latter (318). In SLE there is indeed an increased number of Th17 cells, as well as higher levels of IL17 (319,320).

IL17 is produced by Th17 cells, TCRγδ and TCRαβ double negative T cells, macrophages and neutrophils (318). IL17 induces the production of additional inflammatory cytokines and chemokines, recruiting monocytes and neutrophils to the organs (318). In recent years, several IL17A pathway inhibitors have advanced into clinical trials, including the anti-IL17A monoclonal antibody and the anti-IL17 receptor monoclonal antibody.

The differentiation of the Th17 cells is dependent on IL23 (318). There is also accumulating evidence indicating the importance of IL23 in autoimmune diseases. In a lupus prone mice the clinical and pathology findings were mitigated by IL23 receptor deficiency (321) or by treatment with an anti-IL23 antibody (322). Furthermore,

ustekinumab, a human monoclonal antibody against IL23, has been used with success in the treatment of subacute cutaneous lupus (323).

IL6 is a cytokine with major pro-inflammatory effects, including activation and differentiation of B cells, increased antibody production and differentiation and activation of T cells. IL6 is increased in SLE (324,325) and B cells from SLE patients show upregulation of the IL6 receptor (326). Constitutive expression of IL6 receptors on B cells in conjunction with spontaneous IL6 production by B cells induces autocrine B cell activation, which may lead to B cell hyperactivity and autoantibody secretion in SLE patients (326). The dysregulation of B cell activity observed in patients with SLE may thus be, at least in part, independent of T cell activity (326). The depletion of IL6 through genetic deficiency or antibody-mediated therapy ameliorates disease in mouse models of lupus (327–329).

Finally, it is also noteworthy to discuss the role of IL10 in SLE pathogenesis. Polymorphisms in the *IL10* gene have been linked to SLE risk (330,331).Furthermore, it is known that B cells in SLE patients produce higher levels of IL10, which in an autocrine feedback loop promote B cell proliferation and production of autoantibodies (332). On the contrary, IL10 inhibits T cell function. T cells from SLE produce more IL10 (333), but have a lower expression of the IL10 receptor. T cells from SLE patients are, therefore, more resistant to IL10 (334), which may contribute to its activation.

In conclusion, there is a profound disruption of the cytokine milieu in SLE that is not only a consequence of the immune dysregulation seen in this disease, but that also participates in its amplification.

2.2.5 Major cell types involved in SLE

a) Neutrophils

Neutrophils display various mechanisms of host defense, including expulsion of antimicrobial peptides, phagocytosis of the microorganism followed by their degradation via acid or reactive oxygen species inside phagolysosomes, and trapping the microorganisms in neutrophil extracellular traps (NETs)(242). The latter, as previously discussed, is a mechanism by which neutrophils extrude their chromatin to immobilize and inactivate pathogens (335).

Neutrophils from SLE patients have various abnormalities in their phenotype and function, including defective phagocytosis (229,336,337), which may contribute to the susceptibility to infection seen in this disease. In addition, the production of reactive oxygen species may also be impaired (338). In one study, neutrophils from SLE patients demonstrated reduced reactive oxygen species production in response to activation *ex vivo*, in a manner that correlated with disease severity and end organ damage (338).

Notably, patients with chronic granulomatous disease, who have a defective NADPH oxidase complex and, therefore, a compromised production of reactive oxygen species, have an increased risk for SLE (339). In a registry report 2.7% of the patients with chronic granulomatous disease developed discoid lupus and 0.5% develop SLE (340).

In addition, in the reactive oxygen species-deficient Ncf1 (m1J) mutated mouse model it was found a type I IFN signature, production of autoantibodies, and glomerulonephritis (341). This connection provides further evidence of a link between defective reactive oxygen species generation and autoimmunity. Interestingly, it was also found that neutrophil apoptosis is aberrant in patients with chronic granulomatous disease due, at least in part, to diminished or delayed exposure of phosphatidyl serine on the surface (342). This is an essential step for the uptake of apoptotic cells by phagocytes and may contribute to the development of autoimmunity seen frequently in chronic granulomatous disease.

Neutrophils may also contribute to the break of self tolerance characteristic of SLE by changing the cytokine milieu in the bone marrow and consequently interfering with the normal B cell development (242). Bone marrow neutrophils produce type I IFN, as well as the B-cell activating cytokines APRIL and BAFF (343). Notably, the majority of SLE patients have an IFN signature in the bone marrow more pronounced than the paired peripheral blood and it correlates with both higher autoantibodies and higher disease activity (343). Pronounced alterations in B cell development occur in SLE in the presence of this changed milieu, including an inhibition in early B cell development and an expansion of B cells at the transitional stage (343).

Patients with SLE have a distinct population of low density granulocytes, which display an activated phenotype (344). When compared to normal density neutrophils, these cells secrete increased levels of type I IFNs, IFNγ and TNF and have impaired

phagocytic potential (344). Low density granulocytes also have increased propensity for NETosis in the absence of added stimulation, as previously mentioned (243).

b) Dendritic cells and monocytes

Dendritic cells have a central role in immunity, not only by presenting the antigens to B and T cells, but also by determining their response. Dendritic cells establish and maintain peripheral tolerance via expression of negative co-stimulatory molecules (345). Dendritic cell dysregulation can, therefore, promote the breakdown of B and T cell tolerance and contribute to autoimmunity (346).

SLE patients have fewer circulating conventional dendritic cells, but increased numbers of plasmacytoid dendritic cells (347). As previously described in this chapter, the plasmacytoid dendritic cells are responsible for type I IFN secretion in response to TLR7 and TLR9 activation (348). Another critical function of these cells is the activation of T cells, which, in turn, promote plasmacytoid dendritic cells maturation in a positive feedback loop (349).

In a lupus mouse model, depletion of dendritic cells generally ameliorated the disease (350). Dendritic cells were crucial for the expansion and differentiation of T cells and for the development of kidney interstitial infiltrates (350).

In normal settings, plasmacytoid dendritic cells are capable of inducing both antigenic and tolerogenic responses. The PD-L1 glycoprotein, expressed on the surface of antigen presenting cells in response to stimuli, is a negative co-stimulatory molecule with a tolerizing effect on T cells, mediated by binding to the PD-1 receptor. PD-L1 upregulation is abnormal or absent in myeloid dendritic cells and monocytes from SLE patients during disease flares, suggesting a mechanism by which plasmacytoid dendritic cells may promote T cell autoreactivity rather than tolerance in SLE (345).

Regarding monocytes, several studies support their role in SLE. Monocytes are a key component of the innate immune system, being equipped with sensors designed to recognize pathogen-associated molecular patterns (PAMPs). Activation of these receptors, as previously described in this chapter, leads to the production of a wide-spectrum of cytokines and chemokines. Monocytes can, therefore, initiate inflammation and recruit other immune cells. In addition, monocytes also serve as direct precursors to tissue macrophages, which possess potent phagocytic activity essential for the clearance

of dead cells, cellular debris, microbes, and other foreign material (351). Finally, monocytes have a central role in lupus nephritis and atherosclerosis, two of the main causes of morbidity and mortality in this group of patients.

Regarding nephritis, in SLE there is a milieu that favors kidney leucocyte invasion, since monocytes display elevated surface levels of sialoadhesin and increased urinary levels of ICAM1 and VCAM1 were found in lupus nephritis patients (352). Furthermore, the accumulation of $CD16^{+}$ monocytes in the glomeruli of patients with active lupus nephritis correlates with impaired renal function and anti-dsDNA autoantibodies levels (353). Interestingly, type I IFN increases the expression of sialoadhesin (Siglec-1, CD169) on monocytes, contributing, therefore, to tissue invasion (354).

Monocytes have FcγRs, which participate in phagocytosis, cytolysis, degranulation, and induction of inflammatory cytokines (355). The outcome of interactions with immune complexes is determined by the balance of activating and inhibitory FcγRs on the cell surface of monocytes (356). Notably, the activating receptor FcγRI (CD64) is over-expressed in monocytes from SLE patients, particularly in those with renal disease (357). The elevated levels of type I IFN also contribute for the over-expression of FcγRI in monocytes from SLE patients (356).

Deletion of the Fc receptor γ-chain, an essential signaling component of activating FcγRs, is sufficient to inhibit the development of glomerulonephritis in lupus-prone mice (358). Gamma chain-deficient NZB/NZW mice generated and deposited immune complexes and activated complement, but were protected from severe nephritis, thus showing that there is an uncoupling of immune complex formation and kidney damage in lupus nephritis when FcγRs are inactivated (358). A later study showed that the presence of Fc γ chain on monocytes, but not renal mesangial cells, is required for this effect (359). In humans, polymorphisms of both activating and inhibitory FcγRs are implicated in the development of SLE (356). These will be further discussed in Chapter 3.

FcγR mediated uptake of nucleic acid-containing immune complexes may result in translocation of the endogenous nucleic acids into the endosomal compartment containing TLR 7/8 and TLR9 (356). This mechanism may be responsible for the excess production of inflammatory cytokines by monocytes including IL6, IL10 and BAFF (356). Moreover, the increased ratio of activating to inhibitory FcγRs and the delayed removal of endogenous nucleic acids secondary to deficiency of serum nucleases in SLE patients

may contribute to this effect (356).

In addition, monocytes from SLE patients express greater levels of the costimulatory molecule CD40 (356). Concomitantly, CD40L expression is elevated on T and B cells from SLE patients with active disease (360). Interaction between CD40 and CD40L triggers lymphocyte activation, proliferation, and initiation of immunoglobulin isotype switch (356). Accordingly, the excess of CD40-CD40L interactions may promote the proliferation of autoreactive lymphocytes and production of autoantibodies.

Monocytes also contribute to the intrinsic defect in the clearance of dying cells seen in SLE. *In vitro*, phagocytosis of autologous apoptotic cells is significantly impaired in monocyte-derived macrophages from SLE patients (356). Despite normal surface binding of apoptotic cells, macrophages from SLE patients display reduced ability to internalize the targets (356), a defect due, at least in part, to the reduced surface expression of CD44 (234). Furthermore, addition of serum from SLE patients to macrophages from healthy controls reduces the uptake of apoptotic cells (361).

In conclusion, in SLE aberrant monocyte function contributes to its pathogenesis through impaired removal of apoptotic cells, increased release of pro-inflammatory cytokines, activation of T cells, increased transendothelial migration and tissue damage.

c) T cells

Depletion of T cells or blocking T cell activation mitigates the development of nephritis in lupus prone mice (362), which clearly shows that these cells are important players in SLE pathogenesis (318). One of the first phenomena to be described was the aberrant signaling through the TCR. In T cells from SLE patients the TCR/CD3 zeta chain is downregulated and replaced by FcRγ. FcRγ pairs with Syk rather than ZAP-70, resulting in a hyperactive TCR signaling pathway (363,364). The downregulation of TCR/CD3 zeta chain is not cell intrinsic and can be induced in normal T cells by SLE serum. This phenomenon is influenced by mTOR activation, since treatment of T cells from SLE patients with rapamycin reverses this effect (365).

Despite this phenotype of increased activation, T cell production of IL2 in response to stimulation is actually reduced, as previously described in this chapter. Increased activity of CaMKIV contributes to the elevated levels of CREMα, which binds to

the IL2 promoter and actively suppresses IL2 expression (313,314). Notably, CaMKIV activity can be induced in normal T cells by serum IgG from SLE patients (314).

The reduced levels of IL2 contribute to the imbalance between Th17 cells and regulatory T cells seen in SLE, since IL2 is necessary for the maintenance of regulatory T cells and for the inhibition of th17 differentiation, as previously discussed.

Th17 cells are a subset of $CD4^+$ T cells characterized by higher expression of the pro-inflammatory cytokine IL17. CaMK4 and ROCK induce Th17 differentiation through mTORC1 signaling. Recent studies have shown that the blockade by rapamycin reverses the expansion of Th17 cells in SLE patients (366) and that rapamycin blocks glomerulonephritis in lupus prone mice (318).

$CD3^+CD4^-CD8^-$ double-negative T cells are also expanded in SLE patients (367) and contribute as well to the IL17 production (368). Cells expressing IL17 have been found infiltrating the kidneys of patients with lupus nephritis (367). Double negative T cells also express other pro-inflammatory cytokines, including IL1β and IFNγ, and promote B cell differentiation and antibody production. In a lupus murine model, CXCR5 is important for pathogenic double negative T cells infiltration into lymphoid organs and kidneys (369).

It is also worth mentioning that in SLE there is also an expansion of T follicular helper cells, which correlates with increased disease activity. These cells support B cell differentiation and antibody production (370,371). Recent evidence suggests that the expansion of T follicular helper cells in SLE is directed by the interaction OX40-OX40L, the latter expressed on myeloid antigen presenting cells and induced by TLR7 activation (372).

Finally, recent studies have focused on T-B cell interactions. T cells represent a key checkpoint for autoreactive B cells. In SLE T-B interactions occur outside the usual locations in secondary lymphoid organs and the interactions are more transient, suggesting that the very essence of the interaction is pathologic (373,374).

d) B cells

B cell abnormalities play a crucial role in SLE pathogenesis, since autoantibodies directed against nuclear antigens are near-universally detected in SLE patients.

Usually autoreactive B cells emerge stochastically during the process of somatic hypermutation and suffer apoptosis. In SLE the autoreactive B cells encounter

autoreactive follicular Th cells. This fosters the formation of autoreactive long-lived plasma cells and, consequently, autoantibody production (375).

In addition, SLE patients have increased immature and transitional B cells (375), which show higher levels of polyreactivity and autoreactivity, possibly due to inefficient checkpoint regulation (376). Infection may influence this process, since the maturation of B cells depends on environmental stimuli, including infection, which appears to increase autoimmunity. Murine models, for instance, have demonstrated that transitional B cells are susceptible to accelerated maturation by TLR9, which bypasses tolerance checkpoints (377). Terminal differentiation into antibody-secreting cell associated with isotype switch commitment is also triggered by TLR9 which leads to a striking production of autoantibodies (377). Tolerance can also be broken by B cell stimulation via cytokines, including BAFF. BAFF antagonism in mice clearly leads to improved tolerance to self and conversely BAFF overexpression leads to autoimmunity, as previously mentioned (378,379).

In SLE the function of regulatory B cells is impaired (380), namely induced regulatory B cells from SLE patients are less effective in the control of Th cell proliferation (380). The malfunctioning SLE regulatory B cells might allow the overstimulation of immune responses and contribute to the initiation and perpetuation of disease (380).

Recently, it was shown that a regulatory feedback between plasmacytoid dendritic cells and regulatory B cells is altered in SLE (381). In healthy individuals, plasmacytoid dendritic cells drive the differentiation of immature B cells into regulatory B cells and plasmablasts. A negative feedback loop exists, since regulatory B cells release IL10, controlling plasmacytoid dendritic cells. In SLE, this cross-talk is aberrant (381). There is evidence that plasmacytoid dendritic cells promote plasmablast differentiation, but do not induce regulatory B cells (381). Interestingly, the plasmacytoid dendritic cells – regulatory B cell interactions seem to be normalized in SLE patients responding to rituximab (381).

Autoantibodies contribute to SLE through the formation of immune complexes, which can activate complement and drive inflammation. Antibody production in general appears to favor high affinity in SLE as even anti-influenza antibodies have higher affinity in SLE than in controls (382). A previously unanticipated B cell phenomenon is the production of pathologic IgE antibodies. It was recently found that in SLE IgE antibodies

specific for dsDNA activate plasmacytoid dendritic cells (383). The concentration of dsDNA-specific IgE correlated with disease severity and greatly potentiated the function of plasmacytoid dendritic cells by triggering phagocytosis via the high-affinity FcεRI receptor for IgE, followed by TLR9 activation(383).

Finally, autoantibodies can also directly cause tissue injury. Anti-dsDNA antibodies that cross-react with the NMDA receptor have been found in the serum and cerebrospinal fluid of patients with neuropsychiatric lupus (384). These antibodies may directly cause neuronal cell death, modulate neurosynaptic activity, and impair the integrity of the blood-brain barrier (384–386).

2.3 ORGAN SPECIFIC PATHOGENESIS

New experiments highlight that loss of tolerance and pathology are two different processes and they should be distinguished clinically. Physicians encounter frequently patients with high titers of autoantibodies without evidence of active organ inflammation or damage. Data from mouse model support this concept. Autoimmunity and kidney damage in the NZM2328 lupus-prone mouse are controlled by Agnz1 and Cgnz1, respectively (387,388). Replacement of the pathologic *Cgnz1* variant with the normal allele did not affect the expression of autoimmunity but prevented kidney failure (387). In turn, when the gld.apoE(-/-) mouse, a lupus prone mouse with profound atherosclerosis, was rendered IRF5 deficient, it was protected from autoimmunity, but displayed more atherosclerotic lesions (388). Thus, tissue effects are regulated independently of tolerance. In the next two sections of this chapter the pathogenesis of tissue damage is going to be briefly described.

2.3.1 Lupus Nephritis

Immune complexes activate the local classical complement pathway, as well as kidney macrophages and dendritic cells, causing local release of proinflammatory cytokines and, consequently, intrarenal inflammation and cell injury. The immune complexes are mainly formed by anti-dsDNA antibodies, which bind not only DNA, but also directly to components of the glomerular basement membrane and mesangium (389,390). Immune complexes that are retained on the glomerular basement membrane

fix C1q. The subsequent binding of anti-C1q antibodies to C1q activates complement, resulting in an influx of inflammatory cells (391–394).

The location of the immune complexes determines the lupus nephritis histopathologic class. Large intact immune complexes or anionic antigens, which cannot cross the anionic charge barrier in the glomerular capillary wall, are deposited in the mesangium and subendothelial space (395). Subepithelial deposits form when a cationic antigen is present, which can cross the glomerular basement membrane or when an autoantibody is directed against epithelial cell antigens.

Immune complex deposition in the mesangium causes mesangial cell injury and consequently mesangial cell hyperplasia, a phenomenon characteristic of class II lupus nephritis. Subendothelial immune complex cause endothelial cell swelling, clotting, vascular necrosis, glomerular basement membrane rupture, parietal epithelial cell hyperplasia and crescent formation, which occur in class III and IV lupus nephritis. The typical clinical manifestations are an active urine sediment (red cells, white cells, and cellular and granular casts), proteinuria, and, often, an acute decline in renal function. Subepithelial immune complex deposits injure podocytes, promoting proteinuria and glomeruloesclerosis, characteristic of class V lupus nephritis.

It is hypothesized that a heterogeneous set of genetic variants generates a lupus nephritis cumulative risk. A recent genome-wide association study analyzed SLE women with and without lupus nephritis and found the following gene loci highly enriched in lupus nephritis patients: 4q11-q13 (*PDGFRA, GSX2*), 16p12 (*SLC5A11*), 6p22 (*ID4*), 8q24.12 (*HAS2, SNTB1*) and *HLA-DR2* and *HLA-DR3* (396). The most significant evidence of an association with lupus nephritis was observed with rs1364989, located 83 kb from *PDGFRA.* This gene encodes the α-polypeptide of the PDGF receptor, which is expressed in the glomeruli and in the interstitium and promotes local cell proliferation, extracellular matrix synthesis and cytokine production (397). *PDGF* and *PDGFR* expression is increased in kidney tissue from patients with proliferative forms of glomerulonephritis, including lupus nephritis (398). Furthermore, anti-PDGF antibodies inhibit mesangial cell proliferation in animal models of glomerulonephritis (399).

The association between ethnicity and lupus nephritis risk has also been profusely studied. One example is the identification of two *APOL1* gene variants that are found almost exclusively in African Americans and that have been associated with

glomerulosclerosis and end stage renal disease in a number of disorders including lupus nephritis (400).

The TLRs also have an important role in lupus nephritis. *Tlr4*-deficient mice exposed to pristane produced less inflammatory cytokines, had lower levels of autoantibodies and developed a less aggressive immune complex glomerulonephritis (401). In turn, exposing anti-dsDNA antibody transgenic mice to the TLR4 agonist LPS worsened the kidney disease (402). Furthermore, TLR4 up-regulation at protein or gene level caused increased production of anti-dsDNA antibodies and immune complex-mediated glomerulonephritis (403). In C57BL/6 (lpr/lpr)- TLR2 or TLR4-deficient mice, glomerular IgG deposits and mesangial cell proliferation were significantly decreased and antinuclear, anti-dsDNA and anti-cardiolipin autoantibody titers were dramatically reduced (404).

Mesangial cells also have an important role in lupus nephritis pathogenesis. In a lupus mouse model, danger-associated molecular patterns can activate mesangial cells via the TLR2/MYD88 pathway, increasing the production of IL6 (405). TLR3 signaling also activates human mesangial cells, inducing the expression of CXCL1, a chemokine that attracts neutrophils (406). Intense glomerular expression of CXCL1 was observed in biopsy specimens from patients with lupus nephritis (406). A viral infection can, therefore, trigger a lupus nephritis flare, since viral dsRNA can activate TLR3 in resident renal cells and cause cytokine release (407). In addition, bacterial LPS is also capable of inducing an exacerbation of lupus nephritis (408). TLR4 activation contributes to this effect (409), since, in primary mesangial cells from NZB/W mice, TLR4 activation increases the secretion of osteopontin and MCP1 (409).

Interestingly, female mesangial cells express more TLR3 and respond to TLR ligands with a significantly increased IL6 production. Moreover, in mesangial cells the lack of estrogen receptor alpha significantly decreased IL6 and MCP1 production after activation of TL3, TLR4 and TLR7 (410).

Mesangial cells are also capable of producing large amounts of IFNα. Similarly to what happens in dendritic cells, transfected dsRNA induces IFNα production in mesangial cells. In mesangial cells IFNα seems to establish a proinflammatory loop, since disrupting IFNα signaling dramatically reduced the ability of these cells to produce Il6 (411).

Regarding the role of podocytes, it was found an overexpression of *Tlr8* in

podocytes from BXSB-*Yaa* lupus-prone mice, when compared to control mice. Furthermore, the glomerular levels of *Tlr8* mRNA negatively correlated with the glomerular levels of podocyte functional markers (NPHS1, NPHS2 and SYNPO) and positively correlated with urinary albumin levels (412). Podocytes and glomerular endothelial cells also express TLR2, a pathway activated by circulating bacterial wall components, which was found to increase proteinuria in MRL/lpr mice (413). Finally, TLR9 was also detected in podocytes of children with active lupus nephritis, disappearing during remission (414). TLR9 levels correlated positively with anti-dsDNA antibodies and proteinuria and negatively with nephrin, podocin and synaptopodin (414).

In NZB/W mice and in patients with lupus nephritis, proximal tubular cells expressed TLR9 and its levels correlated with tubulointerstitial injury (415). DNA containing immune complexes purified from the sera of SLE patients induced TLR9 in cultured proximal tubular cells (415).

In conclusion, in lupus nephritis renal parenchymal cells, including podocytes mesangial cells, endothelial and tubular cells, as well as resident and infiltrating immune cells, express TLRs and their activation contributes to the tissue injury characteristic of the disease.

2.3.2 Neuropsychiatric lupus

Regarding the pathogenesis of the central nervous system involvement in SLE, the precise mechanisms remain elusive. A recent meta-analysis indicated that genes associated with the immune complex clearance, such as the *FcγRIIIa, FcγRIIIb* and *ITGAM*, are associated with neuropsychiatric lupus (416). Polymorphisms in the *TREX1* gene, which encodes DNase III, have also been associated with central nervous system involvement susceptibility, particularly with seizures (417).

A central mechanism of neuropsychiatric lupus is the dysfunction of the blood brain barrier, such that immunoglobulins, cytokines, and immune cells can gain access to the brain tissue. This was demonstrated by the increased levels of immunoglobulins and albumin in the cerebrospinal fluid of MRLlpr/lpr mice (418,419). Moreover, these markers correlate with neurodegeneration in periventricular areas and disease activity (418). Endothelial cell activation and the overexpression of ICAM1, VCAM1 and E-selectin facilitate leukocyte entry into the central nervous system and, consequently, immune-

mediated brain injury (420).

The complement system has a key role in the disruption of the blood brain barrier integrity in SLE. It was found that reactive oxygen species production was increased significantly in endothelial cells treated with lupus serum from MRL/lpr mice (419). Furthermore, increased permeability was detected, monitored by changes in transendothelial electrical resistance and cytoskeletal remodeling. These effects were alleviated by pretreating cells with a C5a receptor antagonist or a C5a antibody (419). In addition, MRL/lpr mice treated with C5a receptor antagonist had reduced neutrophil infiltration, lower ICAM, TNF and iNOS mRNA expression and reduced neuronal apoptosis (419). Finally, the serum of MRL/lpr mice caused increased apoptosis in neurons, whereas serum pretreated with the C5a receptor antagonist did not (419).

Auto-antibodies, including antiphospholipid antibodies (421) and those targeting ribosomal P peptides (421), the NMDA receptor (422), and matrix metalloproteinase 9 (MMP9) (423) have been suggested to participate in neuropsychiatric lupus pathogenesis. The levels of autoantibodies in the cerebrospinal fluid correlate more closely with the manifestation of neuropsychiatric lupus than the serum levels of the same antibodies (424). In MRL lpr/lpr mice levels of brain-reactive antibodies in the cerebrospinal fluid correlated with immobility in the forced swim test (425). Furthermore, these antibodies bounded directly to brain regions related to memory formation and emotional reactivity (425).

Cytokines modify the homeostatic regulatory mechanisms and participate in SLE pathogenesis. TWEAK, for instance, has been associated with neuropsychiatric lupus (426). Through activation of its cognate receptor Fn14, TWEAK activates cellular proliferation, angiogenesis, inflammation and apoptosis (427). MRLlpr/lpr mice had depression-like behavior, impaired cognitive function and anhedonia, manifestations that improved in *Fn14*-deficient mice (426). These mice had significantly decreased cellular infiltrates in the choroid plexus and preserved blood-brain barrier permeability, attributable to lower brain levels of VCAM1/ICAM1 and iNOS. Moreover, these mice had reduced IgG and complement deposition in the brain and neuron degeneration and hippocampal gliosis was significantly decreased (426).

In conclusion, in SLE there is a profound immune dysregulation associated with the loss of tolerance to autoantigens. In spite of SLE being a systemic disease and the

mechanisms of its pathogenesis being globally similar, there are specificities in each organ that is affected. These specificities are now being unveiled.

2.4 THE ROLE OF THE ENVIRONMENT

Imperfect disease concordance between monozygotic twins suggests that environmental factors have a role in SLE (428).

Hormones have been recognized as contributors to the pathogenesis of the disease. Females comprise up to 90% of most SLE cohorts and data support a mechanistic role for estrogen and prolactin (429,430). Regarding estrogen, a recent transgenic mouse model demonstrated that accelerated estrogen receptor signaling could induce a lupus-like disease associated with aberrant DNA methylation of the CD40L promoter. Regarding prolactin, its levels are increased in 15% to 33% of SLE patients. Disease activity correlates with prolactin levels and bromocriptine, a dopamine agonist that inhibits prolactin secretion, has proven therapeutic effect. These findings corroborate the role of hormones in SLE.

Ultraviolet light (UV) is thought to drive apoptosis, providing an immunologic stimulus for an increase in the production of autoantibodies. The subsequent antigen-antibody reaction causes a local inflammatory reaction. Furthermore, after exposure to UVB irradiation there is an increase in the production and release of pro-inflammatory cytokines, including IFNα (431), and reactive oxygen species (432). There is also a possible connection between sunlight and lupus-inducing drugs. For instance, UV light converts propranolol into a proinflammatory aryl hydrocarbon receptor ligand, possibly explaining its association with SLE (433).

Infections have an undoubted impact on SLE. Epstein-Barr virus and cytomegalovirus are considered SLE triggers (434), while *Helicobacter pylori* (435), Hepatitis B virus (436) and parasite infections are thought to be protective (437).

Other data support a role for microbes more generally. LPS is a Gram-negative cell wall component that can activate TLR4. Biomarkers of LPS engagement correlate with disease activity and TLR4 activation promotes lupus in mouse models. This pathway activates expression of CD40 by myeloid cells, which is critical for antigen presentation to

T cells. These data suggest that chronic microbial translocation may contribute to the disease pathogenesis.

Bacterial biofilms represent another mechanism by which microbes interact with the immune system. Amyloid-DNA complexes, found in many biofilms greatly increased autoantibodies in lupus-prone mice (438).

The importance of the microbiome to health in general, and SLE in particular, is gaining interest as a critical facet of the disease process. There is a growing body of evidence indicating a role for the microbiota in the pathogenesis of autoimmune diseases, including inflammatory bowel disease (439), type 1 diabetes mellitus (440), multiple sclerosis (441) and rheumatoid arthritis (442). Data regarding the connection between the microbiota and SLE are still scarce. Recently, the dynamics of gut microbiota in the SLE mouse model MRL/Mp-*Fas*^{lpr} were studied (443). In female lupus mice it was found a marked depletion of *Lactobacilli* and increase of Clostridial species compared to age-matched healthy controls (443). Moreover, dietary strategies that restored the gut colonization improved SLE symptoms (443). A higher level of *Bacteroidetes* was found in lupus-prone SNF1 mice (444). In humans, it was shown that intestinal dysbiosis, characterized by a lower *Firmicutes* to *Bacteroidetes* ratio, was present in women with SLE (445). The mechanism of the effect is not fully understood but certain gut bacteria foster the development of regulatory T cells (446,447). While therapeutic treatment of the microbiome in humans has been limited to infections and inflammatory bowel disease, these studies represent an important proof of concept for pursuing additional studies in SLE patients.

Please refer to **Appendix B.1** for the paper ***New Insights into the Immunopathogenesis of Systemic Lupus Erythematosus***, which was published in *Nature Reviews Rheumatology*. This paper reviews the current model of SLE pathogenesis and covers recent studies that extend this model and offer the potential for novel therapeutic interventions in SLE.

CHAPTER 3

Genetics and Epigenetics of Systemic Lupus Erythematosus

3.1 INTRODUCTION

Epidemiological data indicating a higher concordance ratio between monozygotic twins (24%-69%) compared to dizygotic twins or siblings (2%-5%) have made the role of genetics in SLE indubitable (448). Nevertheless, single gene defects related to lupus-like phenotypes have infrequently been described and patients with monogenic causes of SLE are thought to comprise only about 1% of most adult SLE cohorts. The majority of the identified genetic SLE risk factors are, therefore, common variants, with a modest magnitude of risk, which suggests that different mechanisms contribute to the pathogenesis of this disease, including epigenetic factors, which are just starting to be identified.

The proteins encoded by the SLE-associated genes participate in a multiplicity of mechanisms that were described in the previous chapter. Some SLE susceptibility variants are also associated with other autoimmune diseases, which may reflect common molecular pathways.

The HLA region is the most gene-dense region in the human genome, including 120 functional genes, many of those with a role in immunity (449). This region was identified as the strongest determinant of SLE predisposition in all the genome-wide association studies (GWAS) performed (450–453). Furthermore, variants of HLA-DRB1 were associated with SLE in multiple ethnic backgrounds and an HLA-DR3 polymorphism (rs2187668) seemed to have an impact on the propensity to produce autoantibodies in SLE (454).

Factors related to the X chromosome may help to explain the differences between the genders. Three predisposing gene variants on X chromosome (IRAK1, MECP2, TLR7) were identified. Moreover, there is a gene dose effect, since the prevalence of XXY (Klinefelter's syndrome) is increased 14-fold in men with SLE when compared with the general population of men, whereas XO (Turner syndrome) is underrepresented in women with SLE (455).

In this chapter, we will focus on non-HLA genetic risk factors for lupus. Single-gene defects will be briefly described, followed by a summary of the variants and the broad epigenetic changes that have been associated with SLE.

3.2 SINGLE GENE DEFECTS IN SLE

Single gene defects have been recognized as causing lupus since the 1970s. Specifically, complete deficiencies of C1q, C1r, C1s, C2, and C4 are strongly associated with SLE. A penetrance higher than 90% occurs in C1q, with lower penetrance for C4 (75%) and C2 (10%-30%) (456,457). The role of complement on immune complexes and apoptotic body clearance is thought to be the underlying mechanism responsible for this association. Although partial deficiencies of C4 and Mannose-Binding Lectin have been described as predisposing for SLE (458,459), large-scale studies did not support this finding, so it seems unlikely that they markedly increase the susceptibility to lupus. They may, however, modify the disease phenotype (460).

Less commonly described are the associations of chronic granulomatous disease (CGD) and the carrier state for X-linked CGD with discoid and systemic lupus (461–464), presumably due to an inability to clear apoptotic cells, as described in Chapter 2.

The apoptotic pathway is also affected in autoimmune lymphoproliferative syndrome (ALPS). *FAS* and *FASL* are the genes related to classic ALPS, which have been associated with SLE predisposition (465–467). The mechanism of autoimmunity is not fully understood, but may relate to the excess of cytokines, like IL10 and BAFF, that can break B-cell tolerance.

Finally, approximately 10% of the patients with prolidase deficiency develop lupus (468). Cutaneous manifestations are common, but nearly all of the lupus end-organ effects can be seen. The true mechanism connecting prolidase deficiency to SLE is not known.

3.3 SLE ASSOCIATED VARIANTS

In this section and in Table 3.1 SLE associated variants are listed, divided by their proposed mechanism. The detailed pathophysiology of SLE was presented in Chapter 2.

Pathway	Genes
Function of Immune Cells	
Monocytes and Neutrophils	*FCGR2B, FCGR3A/B, ICAMs, IL10, IRF8, ITGAM.*
B-cells	*AFF1, BANK1, BLK, ETS1, FCGR2B, HLA-DR2, HLA-DR3, IKZF1, IL10, IL21, IRF8, LYN, MSH5, NCF2, PRDM1, PRKCB, RASGRP3.*
T-cells	*AFF1, CD44, CD247, ETS1, FYB, HLA-DR2, HLA-DR3, IKZF1, IL10, IL21, PRDM1, PTPN22, STAT4, TNFSF4, TYK2, UBASH3A.*
Signaling	
Toll-like receptor and IFNα signaling	*ACP5, ELF1, ETS1, IFIH1, IRAK1, IRF5, IRF7/PHRF1, IRF8, PRDM1, STAT4, TLR7, TREX1, TYK2, UBE2L3.*
NFkB signaling	*IRAK1, PRKCB, SLC15A4, TNFAIP3, TNIP1, UBE2L3.*
Other pathways	
Clearance of immune complexes	*C1Q, C1R/C1S, C2, C4A/B, FCGR2A/B, FCGR3A/B, ITGAM.*
Apoptosis and clearance of cellular debris	*ACP5, ATG5, DNASE1, DNASE1L3, FCGR2B, TREX1.*
Production or regulation of reactive oxygen and nitrogen intermediates	*GSR, NDUFS4, NOS1.*
Loci with unknown function	*CLEC16A, JAZF1, PTTG1, PXK, TMEM39A, TNXB, UHRF1BP1, WDFY4, XKR6.*

Table 3.1 – **List of genes whose variants were associated with SLE susceptibility.**

3.3.1 Apoptosis and clearance of nuclear debris

In SLE there is an imbalance of apoptosis and the clearance of nuclear debris, which increases the availability of autoantigens, contributing to autoimmunity. Accordingly, several genes related to these mechanisms have been associated with SLE. One example is *ATG5*. Several variants of this gene, which encodes for a protein that participates in caspase-dependent apoptosis and autophagy, have been described in European SLE patients (452).

Another example is TREX1, which was also mentioned in Chapter 2. TREX1 participates in DNA degradation, granzyme A activated apoptosis and oxidative stress response. *TREX1* null mutations are associated with familial chilblain lupus and the Aicardi-Goutieres syndrome, a rare pediatric neurologic condition featuring an inflammatory encephalopathy, with intracranial calcifications, lymphocyte infiltrates and elevated type I IFN levels in cerebrospinal fluid (276,469). *Trex-1*-deficient mice have a reduced postnatal survival, mainly due to inflammatory myocarditis (470). Notably, certain *TREX1* variants were found to be related to SLE susceptibility (471) and, in a large

case-control study, a *TREX1* haplotype was found to be associated with the risk of neurological manifestations in European SLE patients (472). Since TREX1 metabolizes reverse-transcribed DNA of endogenous retroelements, TREX1 deficiency may trigger autoimmunity through the accumulation of nuclear acids (473).

In addition, mutations in *ACP5*, which encodes a protein that participates in lysosomal digestion, were shown to cause bone dysplasia, as well as an IFNα increase and multiple autoimmune diseases, including SLE (474). Although polymorphisms in *ACP5* have not been identified in GWAS, its major substrate, osteopontin, has been found in several studies as disease associated (475). Finally, in a recent study of patients with African ancestry, several novel associations were found between variants of genes associated with the production of reactive oxygen species and SLE (476). Collectively, these findings demonstrate the critical role of clearing nuclear debris in SLE pathogenesis.

3.3.2 Clearance of immune complexes

Genome-wide analysis and candidate gene association studies of murine lupus models and diverse human populations showed a consistent linkage to 1q21.1-24, a region that includes the receptors that recognize the constant (Fc) portion of immunoglobulin isotypes (FcγRs).

FcγRs can activate (FcγRI, FcγRIIA/C, FcγRIII) or inhibit (FcγRIIB) cellular functions, such as phagocytosis, antibody-dependent cellular cytotoxicity, degranulation, antigen presentation, B-cell activation, cytokine production and immune complex clearance. Numerous single nucleotide polymorphisms (SNP) and copy number variants have been characterized in the FcγR genes. Several of those variants have been associated with an increased risk for SLE. For instance, H131R of *FCGR2A* is a common variant (477) that was shown to have lower affinity for the ligand, leading to a profound decrease on the phagocytosis of IgG2 opsonized particles. The also lower IgG binding *FCGR2A* allele 158F was associated with an increase risk for SLE in Caucasians (478), but not in an African-American population (479). Another example is the single amino acid substitution that occurs on the I232T variant of *FCGR2B*, which was also associated with SLE in Asian populations (480,481), but not in Caucasians (482). Defective signaling by the risk *FCGR2B* variant increases the inflammatory response of macrophages to immune

complexes, reduces the threshold for antigen presentation by dendritic cells and facilitates autoreactive B-cell activation (483), thus contributing for autoimmunity.

FCGR variants are not only associated with disease susceptibility, but also with disease progression and phenotypic features. Variants of *FCGR3A*, for example, were associated with end-stage renal disease in patients with lupus nephritis (484,485).

Finally, copy number variation is common in regions of the genome coding for immune related genes and it is also associated with SLE predisposition, namely a low copy number variation at the *FCGR3B* locus was associated with SLE and it affected the immune complex uptake by neutrophils (486).

Complement has a dual role in SLE. On the one hand, there is clear evidence that complement activation contributes to the pathogenesis of the glomerular injury that occurs in lupus nephritis. On the other hand, complement participates in the clearance of immune complexes and apoptotic bodies. As previously discussed, complete deficiencies of complement are among the strongest known genetic risk factors for SLE. Moreover, genes associated with the regulation of the alternative complement pathway have also been recently found to contribute to SLE risk, namely genes encoding complement factor H regulator (CFHR) and five-related CFHR-proteins (487).

3.3.3 Toll like receptors and IFNα pathway

Type I IFNs (α and β IFN) participate in anti-viral immune responses as key regulators of the proliferation, differentiation, survival and activity of the majority of the immune cells (488). Increased expression of IFNα and its regulated genes has been described in SLE (489–493) and propelled the development of IFNα inhibitors for the control of this disease, as previously mentioned in Chapter 2. A number of variants in the receptors that recognize nucleic acids (TLRs), their regulatory molecules (UBE2L3), downstream transcription factors (IRFs, ETS1) and the IFN signaling pathway itself (TYK2) have been described in association with SLE. This large family of variants is a testament of the importance of this pathway in SLE etiopathogenesis.

TLR activation contributes to the production of type I IFNs, which may explain the solid evidence connecting TLRs to SLE pathogenesis. One of the possible examples is the association between a functional variant of *TLR7* and SLE in an Asian population (494). Other robust SLE associations were found with variations in genes coding for the IFN

regulatory factors (IRF): IRF5, IRF7 and IRF8 (495), the transcription factors downstream of TLRs.

IRF5 is a transcription factor that induces the expression of multiple pro-inflammatory cytokines, including IFNα, TNF, IL6, IL17, IL23, MCP1, and RANTES (496). IRF5 is associated with SLE, as well as other autoimmune diseases, including rheumatoid arthritis, Sjögren syndrome, systemic sclerosis, inflammatory bowel disease and multiple sclerosis (497). The IRF5 locus was implicated in SLE through candidate gene analysis (498) and later confirmed by multiple independent case-control cohorts (499–502) and GWAS (451–454). Several IRF5 insertion and deletion polymorphisms and SNPs have been described in association with increased or decreased levels of IRF5, IFNα and, consequently, SLE susceptibility (503,504). Interestingly, IRF5 is necessary for the development of lupus-like disease in mice, which demonstrates the importance of this transcription factor in SLE pathogenesis (505).

IRF7 variants also contribute for SLE predisposition. An *IRF7* SNP (Q412R) is associated with an increase in IRF7 levels and SLE risk in several ancestral populations (506) and additional *IRF7* risk alleles have been associated with anti-dsDNA antibodies and anti-Sm antibodies (507,508).

UBE2L3 is known to participate in the degradation of TLRs and genetic variations in *UBE2L3* were also identified as predisposing for SLE and other autoimmune diseases (452–454,509,510).

ETS1 is a transcription factor that binds the IFN-stimulated response elements, controlling type I IFN-induced transcription. It also participates in the inhibition of Th17 and B-cell differentiation. Evidence of animal models supports the role of ETS1 in SLE, since *Ets1*-deficient mice develop a lupus-like phenotype, characterized by the production of autoantibodies, glomerulonephritis and local activation of complement (511). In humans, *ETS1* was identified as one of the loci associated with SLE predisposition (453,512,513).

Finally, *TYK2* (tyrosine kinase 2) variants were also associated with higher IFN production, SLE and discoid and subacute lupus (498,514).

3.3.4 NFkB pathway

The NFkB pathway is triggered by multiple stimuli, including TLR activation.

Several genes that participate in NFkB signaling were associated with SLE risk, namely *IRAK1* (515,516), *TNFAIP3* (450,453,517), *TNIP1* (453,509), *SLC15A4* (453) and *PRKCB* (518).

IRAK1 is involved in IFNα and IFNγ induction and is a central regulator of NFkB pathway. Five SNPs spanning *IRAK1,* an X chromosome-encoded gene, were associated with both adult- and childhood-onset SLE, in four different ethnic groups (515).

TNFAIP3 encodes A20, an ubiquitin-editing enzyme, which participates in the termination of NFkB signaling. *TNFAIP3* is an established susceptibility locus for SLE (519,520). Recently, a novel TT>A polymorphic dinucleotide was found to be associated with SLE in subjects of European and Korean ancestry (517). This haplotype resulted in reduced *TNFAIP3* mRNA and A20 protein expression, and the enzyme variant bound a nuclear protein complex, which included NFkB subunits, with reduced avidity (517). This haplotype is, thus, associated with a decreased inhibitory activity of A20, which consequently causes an activation of the NFkB pathway. Furthermore, the role of A20 in NFκB inhibition has been demonstrated in animal models by the development of systemic organ inflammation and death within six weeks of birth in A20 deficient mice (521), and by the existence of a lupus-like phenotype in mice with B lymphocyte specific A20 deletion (522).

3.3.5 Function of monocytes and neutrophils

The role of innate immunity in SLE has been increasingly appreciated. Monocytes play essential roles in SLE pathogenesis, since they participate in lupus nephritis and atherosclerosis, processes responsible for considerable morbidity and mortality in SLE, as described in Chapter 1. Increased interest in neutrophils arose with the description of NETosis. These NETs trap microorganisms, decreasing their ability to spread, facilitate the interaction with neutrophil-derived effector molecules and induce the production of cytokines, such as IFNα. A positive feedback loop occurs, since this cytokine increases NETosis. In SLE, circulating immune complexes activate neutrophils and lead to an increase in the production of NETs. The DNA present in the NETs is protected from nuclease degradation, functioning as autoantigen and potentiating autoimmunity and chronic inflammation. This process was also further discussed in the previous chapter.

Genes coding for proteins related to adhesion and migration of both monocytes and neutrophils have been associated with SLE. ITGAM (CD11b), a protein mainly expressed by macrophages, monocytes and neutrophils, encodes a leucocyte-specific integrin, important in the adherence of neutrophils and monocytes to stimulated endothelium. This receptor also participates in the phagocytosis of complement coated particles and immune complexes, since it is a receptor for iC3b. An association between *ITGAM* variants and SLE susceptibility has been documented in multiple populations (451,452,454,523,524).

3.3.6 B cell function

One of the hallmarks of SLE is the production of autoantibodies and the formation of immune complexes that drive the systemic inflammatory response. B cells are thus key players in the pathogenesis of this disease and the existence of effective drugs that target their function, as belimumab and rituximab, further supports their role in SLE. Numerous genes associated with B cell function and signaling have been found to predispose to SLE (525), including *BLK* (451–453), *BANK1* (454,526) and *LYN* (452,527).

The SLE-risk variants found for *BANK1* affect the regulatory sites and functional domains of the protein and contribute to sustained B cell activation through a change in the intracellular calcium levels (526). LYN, a src-tyrosine kinase, is a binding partner of BANK1, whose variants were also associated with SLE in European-derived individuals, with rs6983130 described as an SLE protective factor (527). The complement receptor 2 (CR2/CD21) is a membrane glycoprotein, mainly expressed on B cells and follicular dendritic cells, that has also been implicated in the tolerance to nuclear self-antigens such as single and dsDNA, chromatin and histones (528). Reduced levels of CR2 have been described in SLE and family-based analysis provided evidence for an association of SNPs in *CR2* and SLE in Caucasian and Chinese populations (529). This association was later confirmed in a case-control study of a European-derived population (530).

NCF2, a cytosolic subunit of the NADPH oxidase, was found to participate in B cell activation and recently it was also implicated in SLE susceptibility (495,509).

IKZF1 is a transcription factor involved in the regulation of lymphocyte differentiation and proliferation, and B cell receptor signaling. It also participates in the control of *STAT4* gene expression. Interestingly, the levels of IKZF1 were found to be

decreased in the serum of SLE patients and, recently, a GWAS identified variants of *IKZF1* associated with SLE in an Asian population (453) .

IL10 is a pivotal cytokine, responsible for globally down-regulating the immune response. Interestingly, IL10 production by monocytes and B cells has been shown to correlate with disease activity in SLE. *IL10* polymorphisms were found to be associated with SLE in multiple populations, including European and Asian (531,532).

3.3.7 T cell function

The role of T cells in the orchestration of the immune response cannot be overstated, so, as expected, several genes implicated in T cell function have also been associated with SLE, including *PTPN22*, *TNFSF4, STAT4* and CD247.

Polymorphisms in *PTPN22*, which encodes LYP, a phosphatase that negatively regulates B and T cell receptor signaling, have been variably reported to affect risk for SLE. A *PTPN22* SNP (rs2476601) was associated with multiple autoimmune diseases, including SLE (533). This association was shown in a GWAS (452) and verified in a replication study (509).

LYP interacts with CSK; polymorphisms in *CSK* are also associated with increased SLE risk. B cells from patients carrying the risk allele show enhanced calcium mobilization and B cell activation in response to BCR cross-linking (534).

TNFSF4 is a co-stimulatory molecule found on the surface of antigen-presenting cells. It binds to the T-cell receptor OX40, contributing to the global activation of T cells, with the exception of regulatory T cells, whose generation and function is inhibited by this signal. Protective and risk haplotypes of *TNFSF4* have been reported for SLE (535).

STAT4 is a key regulator of IL3, IL12, IL17 and IFNα signaling, having, therefore, a critical role in the development of Th1 and Th17 immune responses. Associations with SLE and multiple SNPs located within *STAT4* gene have been found in different ethnicities, including African Americans, Hispanics and Asians (451–454,536,537). There is also evidence of an association with other autoimmune diseases (537).

CD247 is a component of the T-cell receptor – CD3 complex, which is decreased in SLE. Aberrant *CD247* transcript variants were detected in SLE T cells and an association between a *CD247* SNP and SLE was detected on a recent GWAS (538).

3.3.8 Genetic susceptibility for SLE and other autoimmune diseases

The clustering of multiple autoimmune disorders in families created the notion of a common autoimmunity-related genetic background. *PTPN2* is one of those examples, since variants of this gene have been associated with juvenile idiopathic arthritis, rheumatoid arthritis, systemic sclerosis, generalized vitiligo, alopecia areata, type 1 diabetes mellitus, Grave disease, Hashimoto thyroiditis, myasthenia gravis and Addison disease (449). Ramos and collaborators, however, showed that only a partial pleiotropy exists among autoimmune diseases (539). For instance, genes like *ITGAM* and *TNFSF4*, which have been clearly associated with SLE, were not found to be associated with other autoimmune diseases. On the contrary, *IL23R* is associated with several autoimmune diseases, but not SLE. Thus, SLE seems to have a distinct pattern of genetic susceptibility.

3.4 EPIGENETICS OF SLE

The phenotype of a cell is broadly determined by the epigenomic landscape, which modulates gene expression and may serve to perpetuate pathologic mechanisms. The term epigenetic typically refers to the study of durable changes in gene expression that are not accompanied by alterations to the nucleotide sequence. Operationally, epigenetics is a term that refers to DNA methylation, histone modifications, and regulatory RNAs. Epigenetic mechanisms are particularly important for autoimmunity, since the expression of pro-inflammatory genes, like TNF, is regulated at chromatin level (540).

DNA methylation and histone modifications change the chromatin structure to allow or prevent the access of the transcription machinery to DNA. DNA methylation refers to the addition of a methyl group to a cytosine at the 5’ position of the CpG dinucleotide, converting the cytosine to methylcytosine. CpG dinucleotides cluster in CpG islands, regions of more than 200 bases with a GC content of at least 50%, where promoters are often located. DNA methylation can, therefore, cause repression by interfering with the recruitment of transcription factors to the promoter region.

The process of DNA methylation is mediated by methyltransferaces. DNMT1 maintains the methylation status during cell replication, while DNMT3A/B induces *de novo* methylation. DNA demethylation is regulated by the TET family of demethylases.

A very well characterized phenomenon seen in SLE is the hypomethylation of DNA in T cells, causing a state of euchromatin and, consequently, a global activation of transcription, which correlates with disease activity (541). Notably, $CD4^+$ T cells from SLE patients with active disease have more extensive demethylation, but the effect seen during a flare was also seen in quiescent disease, supporting its durability, which can set the character of the disease in each patient. As previously described in Chapter 1, patients tend to re-capitulate their original presentation. The epigenetic echoes of their previous disease may influence, therefore, its subsequent course.

Recently, a genome-wide DNA methylation study of naïve $CD4^+$ T cells from SLE patients and controls found significant hypomethylation in IFN-regulated genes (542). Hypomethylation is, therefore, another mechanism responsible for the characteristic type I IFN hyper-responsiveness seen in lupus T cells.

Another key finding was that drugs known to induce lupus-like features inhibit DNA methylation. Procainamide and hydralazine, for instance, were both found to inhibit the methyltransferase DNMT1, the former directly and the latter through the inhibition of the ERK pathway (543).

DNA methylation is a fascinating topic of study because diet is known to influence DNA methylation and this may be one mechanism by which diet contributes to SLE susceptibility (544).

Regarding histone modifications, there are several types of post-translational modifications and multiple amino acid targets. Acetylation and methylation are the best characterized, but phosphorylation, ubiquitination, SUMOylation, ribosylation, deimination, and proline isomerization also occur. Each modification is regulated by a "writer" and "eraser" enzyme. There are, therefore, histone acetyltransferases, histone methyltransferases, and corresponding eraser enzymes such as histone deacetylases and histone demethylases. Alterations to the histone modification profile alter cellular phenotype due to the profound role of these histone modifications on gene expression.

Comparing to DNA methylation, there has been less attention paid to the study of histone modifications in SLE due mainly to technical reasons, since histone modifications studies require a high numbers of viable cells. Next generation sequencing has been used to define the genome wide changes in histone modifications. ChIP-seq (chromatin immunoprecipitation sequencing) and ATAC-seq (assay for transposase-accessible chromatin sequencing) are the methods used. The former measures the enrichment of a specific histone modification, while the latter measures chromatin accessibility.

Histone modifications were initially analyzed in murine lupus models where histone deacetylase (HDAC) inhibitors were found to improve disease features (545). Furthermore, HDAC6 was found to be overexpressed in MRL/lpr mice and treatment directed at normalizing HDAC6 improved disease features (546). The mechanisms by which these agents worked are controversial because they appear to be highly immunosuppressive.

In humans global histone modifications were measured and were found to be aberrant in the T cells from SLE patients and corrected by treatment with mycophenylate mofetil (547).

H4 acetylation is a histone modification associated with activation of transcription. This epigenomic mechanism was found to be overall increased in monocytes from SLE patients (548). This is concordant with the DNA methylation studies. Both would be predicted to drive over-expression of target genes. Notably, 63% of the genes with a higher H4 acetylation had the potential of IRF1 regulation (548). IRF1 is an IFN-induced weak transcription factor, which regulates the transcription of genes involved in immune modulation. Interestingly, IRF1 can interact with p300 to acetylate histones, which could explain the globally increased H4 acetylation pattern seen in SLE (549). This finding ties, once again, the altered epigenome to the known influence of type I IFNs.

Some histone modifications persist after stimulation, “bookmarking” genes for facilitated re-expression. This feature may also contribute to disease chronicity (550,551).

Regarding enhancers, these were globally altered in SLE monocytes, certainly dictating the altered cell behavior. One of the newer therapeutic efforts in SLE was directed at the epigenome and utilizes inhibitors to BRD4, which is a protein critical for

enhancer function (552). These drugs have been shown to be effective in a murine lupus model, again demonstrating the power of genome-wide approaches to identify novel therapeutic targets (553).

MiRNAs are non-coding RNAs responsible for post-transcriptional gene silencing, by blocking translation or causing mRNA degradation. MiRNAs can, therefore, tune the abundance of multiple mRNAs. These regulatory molecules are involved in essential cell mechanisms, including proliferation, differentiation and apoptosis. MiRNAs also exert control on the immune system, particularly on the maintenance of immunological tolerance, participating in the regulation of T cell selection in the thymus, B cell selection in germinal centers, and development of regulatory T cells.

Changes in miRNAs have been identified in peripheral blood mononuclear cells and renal tissue from patients with SLE. Several of the miRNAs appear to have effects that are central to the lupus process and impact TLR signaling and IFN susceptible genes.

miR-146a, which inhibits type I IFN expression by targeting IRF5 and STAT1 (554), was found to be decreased in SLE (554), contributing, therefore, for the high levels of type I IFN characteristic of this disease. Another example is miR-3148, which was found to modulate the allelic expression of a *TLR7* variant associated with SLE (555). Finally, miR-148a is frequently increased in SLE patients and lupus-prone mice (556). Elevated miR-148a levels promoted the survival of immature B cells by inhibiting the expression of the autoimmune suppressor GADD45α, the tumor suppressor PTEN and the pro-apoptotic protein BIM (556). The overall effect of an increased miR-148a expression was an impaired B cell tolerance (556). The first miRNA therapeutic was approved recently and this field is likely to expand rapidly.

Long non-coding RNAs (lncRNAs) are a diverse group of RNAs and, even though their number is more than twice the number of RNAs associated with coding genes, their nomenclature, categorization and functional study is in its infancy. lncRNAs were originally analyzed in the context of malignancy, but increasing evidence has demonstrated that they are involved in diverse biological processes. New studies will certainly focus on this class of RNAs and, hopefully, we will better understand SLE pathogenesis when their function is unveiled.

Finally, it is also interesting to study transcripts related to retroviruses, which have integrated, mutated, duplicated, and undergone gene conversion such that they

now comprise nearly half of the human genome and exhibit multiple functions. The long interspersed nuclear element-1 (L1) is an autonomous family of retro-elements that can become activated in mammalians. L1 expression is highly regulated and the DNA is usually fully methylated to prevent expression. Recently, L1 levels were demonstrated to be increased in renal tissue from patients with lupus nephritis and its expression correlated with type I interferon signature. Certainly, new studies will focus on this class of RNAs.

The interactions and consequences of these mechanisms are under intense study. Histone modifications and DNA methylation can regulate the expression of miRNAs in SLE, as is the case of miR-142 expression on T cells from lupus patients (557), while miRNAs, like miR-21 and miR-148, which are increased in T cells from SLE patients, decrease the expression of the methyltransferase DNMT1 (558). These findings suggest that the epigenome is globally affected in SLE and that the persistence of the epigenomic changes could lead to a durably aberrant gene expression, contributing to the perpetuation of the disease mechanisms.

3.5 NEW DIRECTIONS

The heritability of SLE has been long recognized. The higher concordance rate between monozygotic twins compared to dizygotic twins and the high sibling recurrence risk ratio all support a strong heritability. The first risk locus identified was that of the MHC and still today, alleles within the MHC constitute the strongest genetic susceptibility for SLE in the general population. This seminal finding supports a disease process in which T cells play a central role since their activation is dependent on MHC.

In the last decade, numerous GWAS have been performed. These use a high throughput technology to analyze hundreds of SNPs and capture genome common variants. Through this approach, the joint effect of many weakly contributing variants across different loci can be studied and gene variants associated with different complex diseases can be identified. Interestingly, variants rarely impact the coding exons and are thought to affect regulatory regions in most cases. Moreover, risk variants may be regulating proximal genes or may be acting at a distance through chromosomal looping.

GWAS are particularly tailored for complex polygenic associations, being drastically more sensitive than family studies. In comparison to linkage analysis and sequencing, however, GWAS have less power in cases of allelic heterogeneity and may be affected by the occurrence of epistasis.

The majority of the variants associated with SLE susceptibility only cause a modest risk increase; so large sample sizes are necessary to find significant variations. Furthermore, since the loci found by this kind of study have a weak additive predictive power for a specific phenotype, their clinic relevance may be small. Finally, occasionally results from GWAS are not replicated across studies and in different populations.

An additional source of information has come from gene expression studies. These early studies were performed on peripheral blood mononuclear cells or whole blood and uniformly identified a set of IFN-stimulated genes. Inflammatory and granulocyte signatures were also seen. Recently, gene expression has been examined in sorted cells from patients of different ancestry, demonstrating that IFN-stimulated genes are identified in each cell population, but the specific genes varied dramatically between cell types. In addition, there was wide variation in gene expression between people of different ancestry. This is an important reminder that our current understanding of racial differences is disappointingly rudimentary. Array studies on T cells have shown changes related to disease activity and clinical subsets. These studies played a pivotal role in our appreciation of the function of type I IFNs and paved the way for therapeutics directed at type I IFNs.

Even though the genetics of SLE has ben unveiled in the past decade, there are still innumerous challenges. Predictive mathematical models integrating the weakly contributing loci will be certainly helpful. In addition, it is necessary to understand how specific genetic variants are responsible for the association and the biological effect. Finally, fine mapping and resequencing studies are necessary, as well as new tools for the analysis of transcriptomics, proteomics and metabolomics (559), with the final goal of being able to risk-stratify patents to truly develop a personalized approach to care.

In conclusion, for most patients the pattern of SLE heritability is not characterized by a single gene with a causal Mendelian effect, but by a multigenic mode of inheritance. Further studies are necessary to understand how the identified susceptibility variants contribute to SLE manifestations. Moreover, the majority of the large-scale studies on

SLE genetics were performed in European and Asian populations. Since SLE is more frequent and more severe in other groups, namely Hispanic and African-American, new studies focusing on these populations are essential. The trajectory of our understanding of the disease pathogenesis has been extraordinarily rapid since the introduction of arrays, genomic approaches and epigenetic strategies. Next generation sequencing efforts and other new technologies are also likely to rapidly advance our knowledge. The era of personalized medicine with genomic data incorporated into diagnosis, prognosis, treatment and adverse event prevention efforts may truly be beginning.

Please refer to **Appendix A.1** for the paper ***Genetics and Epigenetics of Systemic Lupus Erythematosus***, which was published in the journal *Current Rheumatology Reports.*

CHAPTER 4

Global Goals

After a brief review of the clinical manifestations of SLE and of the pathogenesis of the disease, the following chapters are devoted to the original work performed during the PhD.

An epidemiology study was the starting point that helped define the focus of the translational projects. The main goals of this clinical study were:

- To describe the incidence and characteristics of major infections in a cohort of patients with juvenile-onset SLE and
- To identify risk factors for major infection in this group of patients.

This project clearly defined the need for new biomarkers and new treatment strategies with fewer side effects. These conclusions propelled all the subsequent translational studies, which used the modern tools of molecular biology to unveil new clinically relevant pathways.

I focused mainly on two of the major complications of SLE: lupus nephritis and macrophage activation syndrome.

Regarding the lupus nephritis project its main goals were:

- To perform a high throughput analysis of the kidney miRNA signature of lupus nephritis;
- To identify differently expressed miRNAs in lupus nephritis when compared to healthy controls and explore their biological functions *in vitro*;
- To study the role of urinary miRNAs as potentially useful biomarkers in lupus nephritis.

Concerning the macrophage activation syndrome project, the main objectives were:

- To analyze the transcriptome of laser-captured hemophagocytes in a mouse model of hemophagocytic syndrome;

- To study the phenotype of Human hemophagocytes in a diverse cohort of patients with hemophagocytic syndrome.

The ultimate aim was to better understand the role of hemophagocytes.

Finally, another project was developed with the goal of characterizing the transcriptome of monocytes in SLE using next generation sequencing of transcripts. The main objective was to globally understand the qualitative and quantitative dysregulation of gene expression characteristic of SLE.

All these translation projects focused on the transcriptome and epigenome of SLE using different methodologies, with the fundamental goal of better understanding the forces that drive the disease manifestations and that contribute to its chronicity.

CHAPTER 5

Major Infections in Juvenile-Onset Systemic Lupus Erythematosus

5.1 INTRODUCTION

As previously discussed in Chapter 1, in comparison with the general population, patients with SLE have an increased death rate from infection (560). In a multicenter study of 1,000 SLE patients followed over 10 years, infections were the cause of death in 25% of cases (561), with similar results observed in other studies (560,562). Infections and active disease are responsible for the majority of deaths within the first five years of disease, while myocardial infarction and thrombotic events tend to occur later, leading to a bimodal distribution of the causes of death in SLE (561). Infections are also responsible for significant morbidity, resulting in hospitalization in 15% of patients (560). The impact of infection in juvenile-onset SLE would be expected to be even higher, inasmuch as children are especially prone to infections and often have a more aggressive course of SLE requiring more potent immunosuppression (19).

As described in Chapter 2, defects in innate and adaptive immunity contribute to the intrinsic risk of infection that occurs in SLE patients (563). Immunosuppressive agents, which have become the standard for the treatment of major organ involvement in SLE, also increase this risk. Moreover, it has been reported that recurrent major infections predict poorer disease outcome in juvenile-onset SLE (564). Prevention and careful monitoring for infections is thus a major challenge in managing patients with juvenile-onset SLE.

Clinical features and treatment-related factors predisposing juvenile-onset SLE patients to major infection have not been adequately delineated. Furthermore, therapy for patients with juvenile-onset SLE has recently shifted to include increased use of steroid-sparing agents, such as mycophenolate mofetil, and biological agents. New studies were, therefore, needed to clarify the risks of infections associated with different immunosuppressive regimens.

5.2 GOALS

The primary goal of this study was to describe the incidence and characteristics of major infections in a cohort of patients with juvenile-onset SLE. Our secondary aim was to identify factors, at the time of diagnosis or during the course of the disease, associated with major infections.

5.3 METHODS

5.3.1 Study design and patients

A retrospective chart review was performed of the clinical courses of juvenile-onset SLE patients from the time of diagnosis or first encounter until December 2009 or loss to follow-up (1991-2009). The inclusion criteria were: diagnosis of SLE before the age of 18, disease duration ≥ 6 months and, at least three clinical encounters during the period 2007 to 2009 in the Pediatric Rheumatology Division of New York-Presbyterian/Morgan Stanley Children's Hospital - Columbia University Medical Center.

A diagnosis of SLE was defined by the presence of four or more of the 1997 revised ACR criteria for the classification of SLE (46) or when a diagnosis of lupus nephritis was confirmed by renal biopsy (120).

Demographic and clinical information were collected, including: gender; ethnicity; age at disease diagnosis; duration of disease and of follow-up; involvement and timing of renal and neuropsychiatric manifestations; co-morbidities and treatment strategies employed. Renal lesions were categorized according to the WHO classification system for lupus nephritis (120). Lupus activity was assessed, using the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of SLE Disease Activity Index (SLEDAI) (148).

The Systemic Lupus International Collaborating Clinics (SLICC) Damage Index (SDI) score was calculated at the conclusion of the study (565,566). The SDI measures irreversible changes secondary to inflammation, adverse effects of medication and co-morbid conditions, which occurred since the onset of SLE, and are present for at least 6 months. Patients who died were excluded from the analysis of damage.

The Columbia University Medical Center Institutional Review Board granted

ethical approval for this study.

5.3.2 Definition of major infections

Major infections were defined as those requiring treatment with parenteral antimicrobial agents or a course of oral antibiotics lasting one week or more, as previously described (564,567,568). To diagnose an infection, it was necessary to identify a causative agent, or to have a diagnosis made by a pediatric infectious disease specialist, pediatric rheumatologist, or other attending pediatrician based on clinical findings, pathologic, and/or radiologic results.

5.3.3 Statistical analysis

Potential factors associated with major infection were studied, using the Chi-square test and the Mann-Whitney U test. In order to account for the effect of multiple comparisons, the Holm-Bonferroni correction was applied. Multivariate logistic regression was performed using Forward Stepwise Likelihood Ratio (LR) and Enter methods to identify possible confounding or effect-modifying factors, and to estimate adjusted odds ratios (OR) and 95% confidence intervals (95% C.I.). Time free of major infection according to juvenile-onset SLE disease activity and treatment was compared with actuarial analysis and expressed graphically by Kaplan-Meier survival curves. The paired t-test was used to detect differences in SLE activity during and after an infection. P-values less than 0.05 were considered significant. Statistical analysis was performed with IBM SPSS Statistics Version 20.0® (SPSS Inc., Chicago, IL, USA).

5.4 RESULTS

5.4.1 Demographic data

During the study period, 120 patients with juvenile-onset SLE fulfilled the eligibility criteria. The demographic and clinical characteristics of the study population are shown in Table 5.1.

The patients were mainly Hispanic (61; 51%) and African-American (34; 28%). The female to male ratio was 3.4:1. Most (63; 52%) were diagnosed between the ages of 10-15 years and 22 (18%) were diagnosed before 10 years of age. A family history of autoimmune diseases was found in 47 patients (39%).

	Number (%) or Mean ± S.D.
Gender	
Female	93 (78%)
Male	27 (22%)
Ethnicity	
Hispanic	61 (51%)
African-American	34 (28%)
Caucasian	16 (13%)
Other	9 (8%)
Age (years) at jSLE Diagnosis	
<5	5 (4%)
5 ≤ Age < 10	17 (14%)
10 ≤ Age < 15	63 (52%)
≥15	35 (29%)
Disease Activity	
SLEDAI at Diagnosis	9.6±6.5
SLEDAI at time of Major Infection	8.2±5.6
SLEDAI 6M after a Major Infection Episode	5.8±5.7
Disease Manifestations during follow-up	
Leukopenia at diagnosis	8 (7%)
Lymphopenia at diagnosis	77 (64%)
Lupus Nephritis	59 (49%)
Neuropsychiatric Lupus	15 (12%)
Cerebrovascular Accident	6 (5%)
Pericarditis	17 (14%)
Pleuritis	9 (8%)
Pulmonary Hemorrhage	4 (3%)
Persistent Thrombocytopenia	30 (25%)
Macrophage Activation Syndrome	2 (2%)

Table 5.1 – Demographic data and clinical characteristics of juvenile-onset SLE patients.

The mean duration of disease at the end of the study period was 5.3±3.2 years (range 6 months-17 years), while the mean duration of follow-up was 5.0±3.2 years (range 6 months-17 years).

5.4.2 Clinical course of juvenile-onset SLE

At time of diagnosis, the mean SLEDAI was 9.6 ± 6.5, 7% of the patients had leukopenia (white blood cells < 3.0 x 10^9/L) and 64% had lymphopenia (lymphocytes < 1.5 x 10^9/L).

During the course of the disease, lupus nephritis was diagnosed in 59 patients (49%), of whom 48 (81%) had confirmation by biopsy; 35 (73%) had class IV lupus nephritis and three patients developed end-stage renal disease (2.5% of all juvenile-onset

SLE patients). Neuropsychiatric manifestations occurred in 15 patients (12%). Cognitive dysfunction was the most common manifestation, followed by significant headache, seizures, psychosis, and focal neurologic signs. Neurolupus was diagnosed in the first year of the disease in 10 patients (67%). Abnormalities on brain magnetic resonance imaging were documented in 10 patients (67%). Cerebrovascular accidents occurred in 6 patients (5%).

All patients in this cohort were treated with hydroxychloroquine. The mean cumulative prednisone dose was 14.4 ± 18.6g (range: 0-126g). Mycophenolate mofetil was used in 67 (56%), azathioprine in 55 (46%), cyclophosphamide in 44 patients (37%), and rituximab in 6 (5%). Four patients (3%) required plasmapheresis.

Two patients died during the study. The causes of death were disseminated cytomegalovirus infection in one patient and macrophage activation syndrome in the other.

5.4.3 Frequency and types of infections

During the study period, 101 major infections were diagnosed, affecting 44 patients (37%), as shown in Table 5.2. Twenty-three patients (19%) had recurrent episodes of major infections (≥2 episodes). The incidence of major infection was 169/1,000 patient-years of follow-up (C.I. 95%= 139/1,000 - 205/1,000). Major infection occurred on average 4.3±3.1 years after juvenile-onset SLE diagnosis.

The most common major infections were skin and soft tissue infections (n=19 episodes), pneumonias (n=17), urinary tract infections with fever (n=13), sepsis (n=10), and herpes zoster (n=8). The causal agent of infection was established in 49 cases (48%). The majority of the cases of herpes zoster was localized and did not require hospitalization. No cases of *Pneumocystis jiroveci* pneumonia were observed in our cohort.

The SLEDAI at time of infection (8.2±5.6) was significantly higher than that six months following (5.8±5.7) the episode (mean difference 2.4, 95% C.I. 0.8-4; p=0.004). The median SLEDAI at the time of infection with herpes zoster was four and only one patient with zoster had an SLEDAI ≥ 8.

Leukopenia (white blood cells < 3.0 x 10^9/L) was present at time of major infection in 14% of episodes, lymphopenia (lymphocytes < 1.5 x 10^9/L) in 73% and neutropenia (neutrophils < 1.0 x 10^9/L) in 2%.

Types of Major Infection	Number	Causative Organisms (n)
Skin and soft tissue infections	19	*Staphylococcus aureus* 3 Unidentified 16
Pneumonia	17	*Staphylococcus aureus* 1 *Citrobacter koseri* 1 *Prevotella melaninogenica* 1 *Aspergillus fumigatus* 1 *Candida albicans* 1 Unidentified 12
Viral infections	17	Herpes simplex 4 Herpes zoster 8 Cytomegalovirus 1 Influenza 2 Mumps 1 Unidentified 1
Urinary tract infections with fever	13	*Escherichia coli* 6 *Pseudomonas aeruginosa* 2 *Proteus mirabilis* 1 *Klebsiella oxytoca* 1 *Enterococcus faecalis* 1 Unidentified 2
Ear, nose and throat Infections	9	
Sinusitis	3	*Streptococcus pneumoniae* 1 Unidentified 2
Otomastoiditis	3	Unidentified 3
Retropharyngeal abscess	1	*Streptococcus pyogenes* 1
Dental abscess	2	Unidentified 2
Sepsis	10	*Staphylococcus aureus* 1 *Staphylococcus capitis* 1 *Streptococcus pneumoniae* 1 *Salmonella enteritidis* 1 *Klebsiella oxytoca* 1 *Diphtheroids* 1 *Candida albicans* 1 Unidentified 3
Fever and neutropenia	5	*Enterobacter cloacae 1* Unidentified 4
Gastrointestinal infections	4	
Acute gastroenteritis	3	*Salmonella enteritidis* 2 Unidentified 1
Spontaneous peritonitis	1	Unidentified 1
Other infections*	7	

* Other infections: 3 cases of cervical adenitis; 1 case of orbital cellulitis; 1 case of sacroiliitis without identified pathogens; 1 case of meningitis caused by *S. pneumoniae*; 1 case of endocarditis caused by *Serratia marcescens*.

Table 5.2 – Major infections and causative organisms identified.

5.4.4 Factors associated with major infection

Major infection was significantly more likely to occur in patients with more active disease. A C3 level < 90 mg/dl at time of diagnosis was associated with major infection, as shown in Table 5.3. We also found an association between a higher SLEDAI at juvenile-onset SLE diagnosis and major infection, as shown in Table 5.4. No association was found between leukopenia or lymphopenia at diagnosis and major infection.

Disease activity was important not only at diagnosis, but also during the course of SLE, since both renal involvement and neuropsychiatric manifestations of SLE were found to be associated with major infection (Table 5.3).

Uses of cyclophosphamide and mycophenolate mofetil were each associated with major infection in our cohort (Table 5.3). In order to reduce the confounding effect of treatment with both medications, we analyzed the effect of mycophenolate mofetil in the subgroup of patients who had never received cyclophosphamide. In this group as well, treatment with mycophenolate mofetil was associated with major infection (O.R. 4.0; 95% C.I. 1.4-12.0; p=0.01). The association between mycophenolate mofetil and major infection remained statistically significant even after adjusting for the effect of the cumulative dose of prednisone and disease duration (Table 5.3). Finally, the duration of treatment with mycophenolate mofetil was also associated with major infection, with the risk increasing 16% (O.R. 1.2; 95% C.I. 1.0-1.3) after 6 months and 34% after 12 months of treatment with this drug (O.R. 1.3; 95% C.I. 1.1-1.6). On the other hand, neither treatment with azathioprine (Table 5.3) nor its duration of administration increased the occurrence of major infections significantly (Table 5.4).

Use of cyclophosphamide was associated with the occurrence of more severe infections, including sepsis (O.R.=7.0; 95% C.I. 1.4 - 35.4; p=0.012) and pneumonia (O.R.=4.0; 95% C.I. 1.1 - 14.2 p=0.03), while use of mycophenolate mofetil was not.

While variability in use occurred in this cohort, the cumulative dose of prednisone was significantly associated with major infection (Table 5.4). It was estimated that a 10g increase in the cumulative dose of prednisone caused a 40% increase in the risk of major infection (O.R.= 1.40; 95% C.I. 1.04 – 1.88).

Variable	Proportion of patients with an episode of major infection n/N (%)		Crude OR (95% C.I.)	p-value	OR adjusted to cumulative prednisone (95% C.I.)	p-value	OR adjusted to disease duration (95% C.I.)	p-value
Sex	Male: 8/27 (29.6%)	Female: 36/93 (38.7%)	1.5 (0.6-3.8)	0.389	1.3 (0.4-4.3)	0.634	1.4 (0.5-3.6)	0.501
Age at diagnosis	< 10 Yrs: 10/22 (45.4%)	≥ 10 Yrs: 34/98 (34.7%)	1.6 (0.6-4.0)	0.344	1.0 (0.3-4.0)	0.935	1.1 (0.4-3.1)	0.806
C3 at diagnosis	C3 <90 mg/dl: 26/56 (46.4%)	C3 ≥ 90 mg/dl: 8/39 (20.5%)	3.4 (1.3-8.6)	**0.011 ***	3.0 (0.9-10.0)	0.072	2.8 (1.1-7.5)	**0.033**
Lupus nephritis (LN)	No LN: 14/61 (23.0%)	LN: 30/59 (50.8%)	3.5 (1.6-7.6)	**0.004 ***	2.2 (0.8-5.7)	0.108	3.0 (1.3-6.7)	**0.008**
Neuro-psychiatric SLE (NPSLE)	No NPSLE: 32/105 (30.5%)	NPSLE: 12/15 (80.0%)	9.1 (2.4-34.6)	**0.004 ***	5.9 (1.0-34.2)	**0.046**	8.5 (2.2-32.6)	**0.002**
Cyclophos-phamide (CYC)	No CYC: 19/76 (25.0%)	CYC: 25/44 (56.8%)	3.9 (1.8-8.7)	**0.005 ***	2.5 (0.9-6.8)	0.071	3.3 (1.4-7.5)	**0.005**
Mycophe-nolate mofetil (MMF)	No MMF: 11/53 (20.8%)	MMF: 33/67 (49.2%)	3.7 (1.6-8.4)	**0.003 ***	2.9 (1.0-8.3)	**0.041**	3.5 (1.5-8.0)	**0.004**
Azathioprine (AZT)	No AZT: 20/65 (30.8%)	AZT: 24/55 (43.6%)	1.7 (0.8-3.7)	0.145	0.9 (0.3-2.6)	0.835	1.6 (0.7-3.4)	0.245

***** p-values corrected according to the Holm-Bonferroni method. Chi-square tests were used to study the association between variables. Logistic regression was used to determine the adjusted odds ratios.

Table 5.3 – Association between categorical variables and major infection in jSLE patients.

Factor	SLE patients without major infection	SLE patients with major infection	p-value	p-value adjusted to cumulative prednisone	p-value adjusted to disease duration
SLEDAI at diagnosis	8.2 ± 5.9	12.0 ± 6.9	**0.006***	0.242	**0.012**
Duration of treatment with mycophenolate mofetil (months)	10.6 ±20.4	24.3 ±27.4	**0.005***	0.071	**0.039**
Duration of treatment with azathioprine (months)	5.2 ± 10.0	7.7 ± 13.6	0.246	0.764	0.348
Cumulative dose of prednisone (g)	11.0 ± 14.6	21.7 ± 23.9	**0.004***	N/A	**0.011**

* p-values corrected according to the Holm-Bonferroni method.

Independent samples Mann-Whitney U test was used to study the association between variables. Logistic regression was the method performed to calculate the confounding effect of cumulative dose of prednisone and disease duration.

Table 5.4 – Association between continuous variables and major infection in jSLE patients.

We assessed the time free from major infection in the cohort according to disease activity, SLE manifestations, and treatment as shown in the Figure 5.1.

Patients with C3 <90mg/dl at diagnosis or those who developed neuropsychiatric manifestations of SLE during the course of the disease had a significantly lower time free from major infection ($p=0.037$; $p<0.0001$, respectively). Patients treated with mycophenolate mofetil or those with a cumulative dose of prednisone at the end of the study higher than 15g also had a significant decrease on the time free from major infection ($p=0.015$, on both cases).

The variables included in the logistic regression (Forward Stepwise Likelihood Ratio method) were history of lupus nephritis, neuropsychiatric manifestations of the disease, treatment with cyclophosphamide, treatment with mycophenolate mofetil and cumulative dose of prednisone. This analysis demonstrated that the combined effect of treatment with cyclophosphamide and cumulative dose of prednisone was significantly associated with major infection ($p=0.04$).

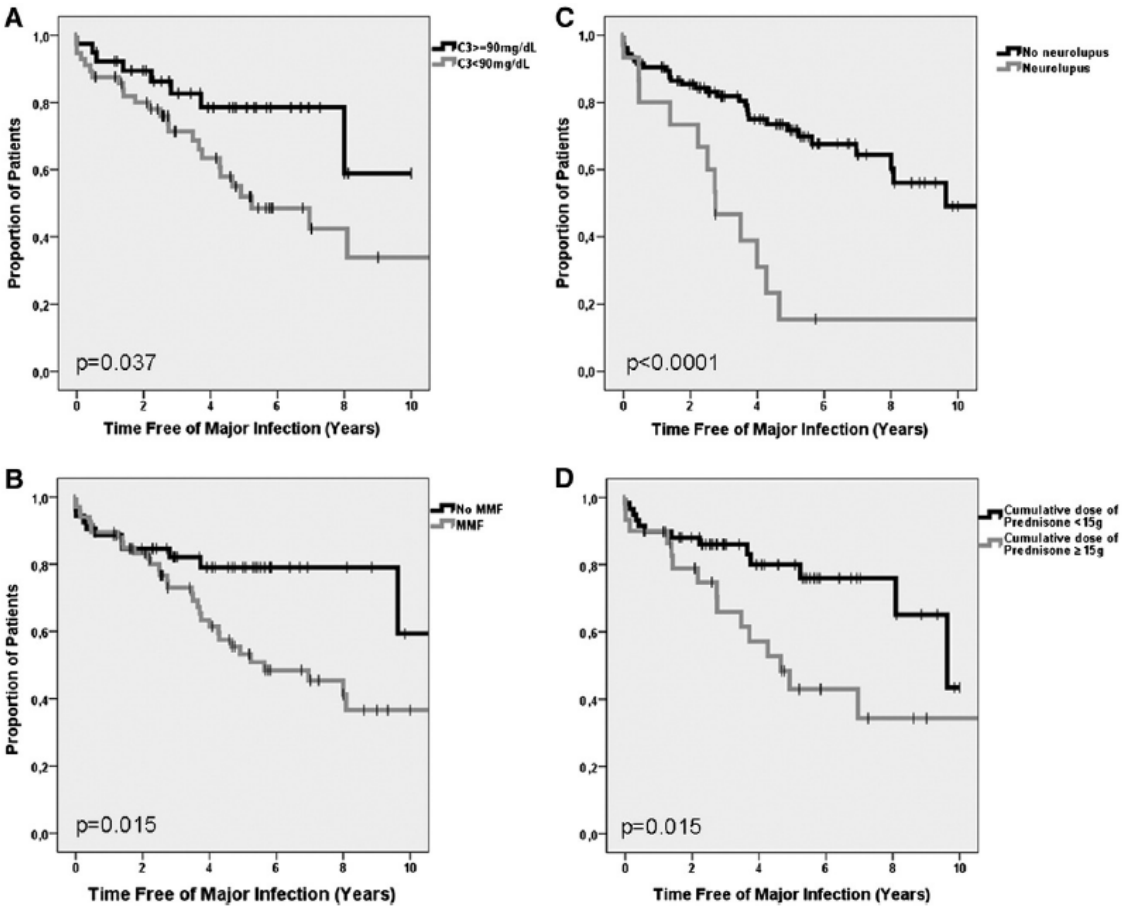


Figure 5.1 – Kaplan-Meier analysis of time free of major infection in patients with juvenile-onset SLE according to disease activity and types of treatment.

5.4.5 Major infection and damage

Damage (SDI ≥1) was present at the conclusion of the study in 33% (39/118) of the surviving patients after a mean disease duration of 5.3±3.2 years. Damage to the musculoskeletal system was observed most frequently (9%), followed by neuropsychiatric (7%), ocular (7%), skin (7%), pulmonary (5%) and renal (5%) systems. Avascular necrosis and osteoporosis with fracture or vertebral collapse were the most frequent types of musculoskeletal damage. No malignancies were encountered.

Major infection was associated with damage (OR=3.2; 95% C.I.=1.4-7.2; p=0.004). This was especially true for patients with sepsis (OR=17.1; 95% C.I.=2.0-144.3; p=0.002).

5.5 DISCUSSION

The remarkable improvement in survival of juvenile-onset SLE patients over the past few decades, reported to be greater than 95% after five years of disease (564,569,570), is likely due to earlier diagnosis, better monitoring of disease activity, and more judicious use of immunosuppressive agents. In this cohort of 120 juvenile-onset SLE patients, two deaths (2%) occurred after a mean disease duration of 5.3 years.

With this increase in life expectancy, patients are faced with morbidities due to sequelae of disease and adverse effects of medication, including major infection. The focus of management has now shifted from prevention of premature death to minimizing permanent organ damage. Identification of risk factors that lead to major infection and long-term damage is therefore of increasing concern. In our cohort, a statistically significant association between major infection and damage was detected. Lee and colleagues had already shown that recurrent major infections were associated with permanent damage in juvenile-onset SLE (564) and Chen and coworkers also found that among SLE patients an episode of bacteremia was associated with an unfavorable long-term outcome (571).

In our study, 37% of patients had at least one episode of major infection. In a prospective and controlled study of another 110 patients with SLE and 220 controls, Bosch and colleagues found that 36% suffered at least one infection versus 22% in the control group, RR 1.63 ($p<0.05$) (572). Others have reported rates of infection ranging from 38%, among adults with SLE, to 57%, among children (564). In a large cohort of 33,565 SLE patients, 7,113 of whom had lupus nephritis, the incidence rate per 100 person-years of serious infections requiring hospitalization was 10.8 in the SLE cohort and 23.9 in the lupus nephritis subcohort (573).

Variation in the rate of infection is most likely due to differences in age groups, duration of follow-up, disease activity, severity of organ involvement and immunosuppressive regimens employed. Furthermore, the definitions of infection have also been heterogeneous, with many studies including minor infections. Despite the variability among the criteria used, the spectrum of infections and causative microorganisms have been similar in adults and children.

Soft tissue infections, pneumonias, urinary tract infections with fever, sepsis and herpes zoster were the most common infections found in our study and reported in the literature (560,564,572,574,575). In addition to unusual opportunistic infections, juvenile-onset SLE patients also experienced infections caused by common pathogens that behaved more aggressively. For example, a case series suggested an increased risk of nontyphoidal *Salmonella* infection in SLE patients, in association with cellular immune defects (576–578). We also documented three cases of nontyphoidal *Salmonella*, including one case of sepsis.

Herpes virus infections are also common. The reported incidence of herpes zoster in SLE ranges from 6.4-16 events/1,000 patient-years, which is remarkably greater than the one reported in the general population (579–581). The incidence found in our cohort was 13 events/1,000 patients-years with a median SLEDAI at the time of herpes zoster of four. These results are in concordance with other studies that showed that herpes zoster usually occurs during periods of quiescent SLE activity (581).

It has been reported that over 90% of SLE patients are seropositive for cytomegalovirus and while overt clinical disease is rare, it carries a high risk of mortality (582–584). In the current study, we found similar results, considering that only one case of cytomegalovirus infection was identified and resulted in the death of the patient.

Infections caused by Parvovirus B19 and Epstein-Barr virus were not documented in our cohort, but potentially were underdiagnosed.

We did not encounter any case of *Pneumocystis jiroveci* infection, even though we do not routinely provide prophylaxis against this organism. Gupta *et al.* have also shown that the number of reported infections with this agent in SLE patients treated with cyclophosphamide is very low (15.88/10,000 patients)(585). Our data complements, therefore, the growing body of evidence against the need for prophylaxis against *Pneumocystis jiroveci* in juvenile-onset SLE.

Disease activity was clearly associated with the occurrence of major infection in this study, as a higher SLEDAI at diagnosis, renal involvement, and neuropsychiatric manifestations were all identified in univariate analysis as factors associated with major infection. Others have found lung and renal involvement (573,574) and SLEDAI > 12 at diagnosis to be predictors of infection (567). In addition, similar to our findings, low

complement levels (C3 < 90mg/dl) have been documented as predictors of major infection (567,572).

In our cohort, use of cyclophosphamide was associated with major infection. Although Bosch and colleagues also documented this association (572), it has not been consistently noted by others (560,567).

We found that mycophenolate mofetil treatment was strongly associated with major infection in juvenile-onset SLE, even in the subgroup of patients who had never been treated with cyclophosphamide and after adjusting for the effects of cumulative dose of prednisone and disease duration. Furthermore, the duration of treatment with mycophenolate mofetil was also found to be a risk factor for major infection in the univariate analysis (p=0.005). On the other hand, azathioprine treatment and its duration were not associated with major infection.

The cumulative dose of prednisone was associated with major infection in our cohort as described in other series (567,572,573). Ruiz-Irastorza and colleagues concluded that an increase of 10 mg per day of prednisone caused an 11-fold increase in the risk of serious infection (574).

In this cohort, according to multivariate analysis, the combined effect of treatment with cyclophosphamide and cumulative dose of prednisone was strongly associated with major infection, which once again supports the need of a thoughtful use of these drugs.

This study had limitations. This was a retrospective study with the potential for missing data and lack of standardized treatment. Our center serves as a tertiary referral center, which most likely increases the severity of disease. Moreover, in our cohort the majority of the patients are Hispanic or African-American, which represent groups known to have more severe courses of juvenile-onset SLE, and might not be generalizable to other centers. We could not assess the effect of hydroxychloroquine on major infections, as all patients in our cohort were treated with this agent, nor could we assess the effect of rituximab due to the small number of patients treated with this agent prior to 2009. Finally, many variables (e.g., lupus activity, hypocomplementemia, renal disease, neuropsychiatric manifestations, treatment with corticosteroids and other immunosuppressive drugs) are inter-related in clinical practice, and their relative weight and interactions are difficult to determine, even with multivariate analysis.

Finally, the relationship between SLE and infections is complex. Not only can active disease lead to infection, but infections can also act as environmental triggers that induce or promote SLE flares. In our cohort, we found that the disease was clearly more active at the time of infection when compared to a clinical encounter six months later.

5.6 CONCLUSIONS

In this large cohort of urban North American children and adolescents with juvenile-onset SLE, major infections were found to be common, were associated with active disease and its treatment, and resulted in noteworthy morbidity. In order to ensure optimal patient outcomes, it is essential to perform regular surveillance for infections, with frequent visits, particularly in patients with more active disease and undergoing treatment with immunosuppressive drugs, and to promptly initiate treatment on presumption of infection. In a recent review of patients with SLE admitted to an Intensive Care Unit, a delay in adequate antimicrobial therapy of more than 24 hours was associated with increased mortality (586).

Lastly, many infections can be prevented with timely immunization, reducing exposure to contagious contacts, screening for latent infection, and minimizing exposure to immunosuppressive drugs, which are essential to achieve optimal control of SLE activity, but the judicious use of which is equally important to avoid infections and other associated adverse effects (587). These measures will help to reduce the burden of major infections in juvenile-onset SLE patients and consequently improve patient outcomes. New biomarkers to guide the use of immunosuppressive drugs and new treatment strategies with fewer side effects are urgently needed. The study of the pathophysiology of SLE will undoubtedly help to identify new clinical relevant pathways.

Please refer to **Appendix A.2** for the paper ***Major Infections in a cohort of 120 patients with juvenile-onset systemic lupus erythematosus***, which was published in the journal *Clinical Immunology*.

CHAPTER 6

The Role of microRNAs in Lupus Nephritis

6.1 INTRODUCTION

The profound impact of lupus nephritis on the outcomes and quality of life of SLE patients led me to pursue the study of its pathogenesis. I was particularly interested in exploring the kidney miRNA pattern of lupus nephritis in order to identify new clinically relevant pathways.

As previously described in Chapter 3, cellular miRNAs are single-stranded, non-coding, RNAs, which have a key role in the post-transcriptional regulation of gene expression. pri-miRNAs are long primary transcripts that undergo sequential processing by the endonucleases Drosha and Dicer in order to become mature miRNAs (~20-23 nucleotides). These guide the RNA-induced silencing complex (RISC) to mRNA targets by imperfect complementary base pairing, mostly at the 3'-untranslated region (3'-UTR). The target mRNAs undergo accelerated turnover or translational repression, causing consequently the silencing of the gene. Each miRNA can regulate hundreds of target mRNAs and thereby control almost every biological pathway (588). MiRNA dysregulation can globally interfere with gene expression and be associated with a dramatic change in cell behavior and, consequently, with human diseases. It is not surprising, therefore, the growing body of evidence reporting miRNA dysregulation in human diseases. The development of high-throughput methodologies for the global measurement of miRNAs, and the stability of these molecules in biologic fluids, allowed them to emerge as a new class of biomarkers.

The previous studies of the SLE miRNA pattern have identified miRNAs with aberrant expression associated with different mechanisms known to participate in the disease pathogenesis, including innate immunity and type I IFN signaling, inflammation and dysregulation of apoptosis (589–591). It was identified in SLE, for instance, a dysregulation of miR-146a, which is involved in the control of type I IFN signaling (592). Another example is miR-125a, which is down-regulated in SLE. One of its targets is KLF13, whose levels are increased, as expected, with the down-regulation of miR-125a. This leads to an increase of RANTES and, consequently, to inflammation (593).

The down-regulation of miR-181a, which was reported in pediatric SLE (594), causes an increase of one of its targets P300/CBP-associated factor (PCAF), inducing apoptosis (594).

In SLE miRNA dysregulation is also associated with changes in other epigenetic control mechanisms, including DNA methylation and histone modifications. One example of this type of interaction in SLE is miR-126, which is overexpressed in $CD4^+$ T cells of SLE patients. This decreases the expression of the methyltransferase DNMT1 and causes *CD11A* and *CD70* demethylation, contributing to T and B cell hyperactivity (595). miR-21 is also up-regulated in $CD4^+$ T cells of SLE patients and lupus-prone MRL/lpr mice and its expression correlates with disease activity (596). miR-21 represses *DNMT1* as well, but indirectly through the down-regulation of RasGRP1. miR-21 up-regulation is, therefore, also associated with hypomethylation. Notably, procainamide and hydralazine, two drugs known to induce lupus-like syndromes, were both found to inhibit DNMT1, which shows the importance of DNA hypomethylation and global activation of transcription in SLE etiopathogenesis, as previously described in Chapter 2.

Several studies of miRNAs in SLE have been performed in plasma (597), peripheral blood mononucleated cells (598–600) and in the kidneys of lupus nephritis patients (601,602). However, they have been limited and have not used an unbiased, high throughput approach. Further studies are, therefore, necessary to better understand miRNA dysregulation in lupus nephritis. This is important considering that lupus nephritis is still a challenging disease, particularly in children, being associated with considerable morbidity and mortality (55). It is estimated that up to 20% of children with proliferative lupus nephritis progress to renal failure over a 10-year period, which is significant in a young population (603).

In addition, the definitive diagnosis of lupus nephritis is still based on renal biopsy findings, which have modest predictive value for outcome, as formerly discussed in Chapter 1. Noninvasive strategies are thus needed for both lupus nephritis diagnosis and monitoring. Furthermore, the use of immunosuppressive drugs, which have become the gold standard for the treatment of lupus nephritis (173), is particularly contentious, since they are associated with several side-effects, including the occurrence of major infections (604), as previously shown in Chapter 5, and infertility (605,606). The identification of new biomarkers to guide the use of these therapeutic agents and the development of new treatment strategies with fewer side effects would have, therefore, an enormous impact in the management of lupus nephritis patients.

6.2 GOALS

The main goal of this study was to analyze the miRNA pattern of the kidneys of patients with lupus nephritis and to study the role of the dysregulated miRNAs in the pathogenesis of the disease, with the ultimate aim of identifying new clinically relevant pathways.

6.3 METHODS

6.3.1 Patients and kidney specimens

Paraffin-embedded kidney samples were selected from pediatric patients with lupus nephritis or post-streptococcal glomerulonephritides. The controls were normal kidney samples from adult donors and one sample of normal kidney tissue from a child with nephroblastoma. Table 6.1 summarizes the demographic and clinical characteristics of the lupus nephritis cohort. A Pathologist confirmed the diagnoses and classified the lupus nephritis findings, according to the International Society of Nephrology and the Renal Pathology Society criteria (120). The institutional review board of The Children's Hospital of Philadelphia approved the study.

6.3.2 RNA extraction and miRNA quantification

RNA was extracted with the formalin-fixed paraffin-embedded miRNeasy kit (Qiagen) and analyzed using a NanoDrop 2000 spectrophotomer. High purity samples were chosen, according to the absorbance ratios 260:280 and 260:230. Seven hundred thirty-four miRNAs were analyzed by direct digital detection of molecular barcodes, with the nCounter assay (NanoString). This method is ideal for fragmented RNA samples and is highly sensitive for miRNAs. Six negative miRNA assay controls, six positive miRNA spikes and five housekeeping mRNA controls (*ACTB*, *B2M*, *GAPDH*, *RPL19* and *RPL0*) were also quantified. The data were normalized to the sum of the six positive control miRNA spikes. To account for differences in miRNA content in each sample, the data were normalized to all miRNA counts for each assay. Principal component analysis was performed using the R statistical computing language (www.r-project.org) and pathway analysis was done with

Ingenuity. Statistical significance was considered when the Benjamini-Hochberg false discovery rate was below 0.05.

Characteristics	Number (%) or Mean ± S.D.
Gender	
Female	8 (67%)
Male	4 (33%)
Ethnicity	
African-American	5 (42%)
Asian	2 (17%)
Caucasian	3 (25%)
Hispanic	2 (17%)
Age at SLE diagnosis (years)	13 ± 2
Age at lupus nephritis diagnosis (years)	14 ± 3
Age at time of kidney biopsy (years)	14 ± 3
Biopsy during the first year after diagnosis	11 (92%)
SLE manifestations until the time of kidney biopsy	
Malar rash	4 (33%)
Oral ulcers	2 (17%)
Non-erosive arthritis	7 (58%)
Pleuritis or pericarditis	1 (8%)
Renal disorder	12 (100%)
Neurological disorder	1 (8%)
Hematological disorder	8 (67%)
Immunologic disorder	10 (83%)
Anti-nuclear antibody	12 (100%)
Antibody Profile	
Anti-nuclear antibody	12 (100%)
Anti-dsDNA	9 (75%)
Anti-Sm	3 (25%)
Anti-RNP	4 (33%)
Anti-SSA	2 (17%)
Anti-SSB	1 (8%)
Anti-phospholipids antibodies	2 (17%)
SELENA-SLEDAI at time of Kidney biopsy	17 ± 7
Renal SELENA – SLEDAI at time of kidney biopsy	11 ± 4
Urinary casts	9 (75%)
Hematuria	8 (67%)
Proteinuria	10 (83%)
Pyuria	3 (25%)
Lupus nephritis active at time of kidney biopsy	12 (100%)
Treatment at time of kidney biopsy	
No treatment	8 (67%)
Hydroxychloroquine	2 (17%)
Prednisone	4 (33%)
Mycophenolate mofetil	1 (8%)
Azathioprine	0 (0%)
Cyclophosphamide	3 (25%)
Rituximab	0 (0%)

Table 6.1 – Demographic and clinical information regarding the patients whose samples were used to study the kidney miRNA pattern.

The miR-26a and miR-30b levels were validated in the same cohort by quantitative real-time polymerase chain reaction (qRT-PCR), using Taqman miRNA assays (Applied Biosystems) and the 7900HT Fast Real-Time PCR System. Relative quantification was applied using spiked *Caenorhabditis elegans* (*C. elegans*) miRNA-238 as the internal control (Qiagen). Commercially available primers were purchased from Applied Biosystems (Table 6.2).

Name	Company	Catalog Number	Code
hsa-miR-26a	Applied Biosystems	4427975	000405
hsa-miR-30b	Applied Biosystems	4427975	000602
cel-miR-238	Applied Biosystems	4427975	000248

Table 6.2 – Primers for miRNA quantification.

6.3.3 Patients and urine specimens

Urine samples were collected from healthy individuals and adult patients followed at the Hospital of the University of Pennsylvania. All of the patients had demonstrated clinical and laboratory evidence of active lupus nephritis (hematuria, proteinuria, pyuria and/or urinary casts) at one or more encounters in the past three years. Table 6.3 displays the characteristics of this cohort.

The urine samples were centrifuged at 3,000 revolutions per minute at 4°C for 30 min and aliquots of 100µL of the supernatants with 500 µL of QIAzol lysis reagent were kept at -80°C. RNA was extracted from these samples using Qiagen miRNeasy serum/plasma kit. miR-26a and miR-30b were measured by qRT-PCR, as previously described. The IRBs of the Children's Hospital of Philadelphia and of the University of Pennsylvania approved the study and patients and controls provided informed consent for their participation.

Characteristics	Number (%) or Mean ± S.D.
Gender	
Female	12 (86%)
Male	2 (14%)
Ethnicity	
African-American	8 (57%)
Caucasian	4 (29%)
Asian	1 (7%)
Hispanic	1 (7%)
Age at SLE diagnosis (years)	20 ± 11
Age at lupus nephritis diagnosis (years)	23 ± 13
Age at time of urine collection (years)	37 ± 10
Time between lupus nephritis diagnosis and urine collection (years)	14 ± 10
Clinical and laboratory manifestations of SLE	
Malar Rash	8 (57%)
Discoid Rash	5 (36%)
Photosensitivity	3 (21%)
Oral ulcers	4 (29%)
Arthritis	11 (79%)
Serositis	2 (14%)
Renal disorder	14 (100%)
Neurologic disorder	5 (36%)
Hematologic disorder	6 (43%)
Immunologic disorder	11 (86%)
Positive anti-nuclear antibody	14 (100%)
Antibody Profile	
Anti-dsDNA	9 (71%)
Anti-Sm	3 (21%)
Anti-RNP	5 (36%)
Anti-SSA	4 (29%)
Anti-SSB	0 (0%)
Anti-phospholipids antibodies	1 (7%)
Kidney biopsy	10 (71%)
Lupus Nephritis Class IV	6 (60%)
Lupus Nephritis Class II and V	2 (20%)
Lupus Nephritis Class III and V	1 (10%)
Lupus Nephritis Class IV and V	1 (10%)
SELENA-SLEDAI at time of urine collection	4 ± 4
Patients with active lupus nephritis at time of urine collection	4 (29%)
Renal SLEDAI of the patients with active lupus nephritis at time of urine collection	6 ± 2
Treatment at time of urine collection	
Hydroxychloroquine	10 (71%)
Prednisone	7 (50%)
Mycophenolate mofetil	10 (71%)
Azathioprine	0 (0%)
Cyclophosphamide	0 (0%)
Rituximab	0 (0%)
Methotrexate	1 (7%)

Table 6.3 – **Demographic and clinical information regarding the cohort used to study miRNAs in the urine.**

6.3.4 Cell Lines

Human renal mesangial cells (ScienCell), Human Kidney-2 cells (ATCC) and human podocytes, kindly provided by Dr. Holzman at the University of Pennsylvania, were maintained according to the manufacturer's recommendations. Human mesangial cells were selected for the majority of the *in vitro* studies, since the proliferation of this type of cell is an important phenomenon of lupus nephritis pathogenesis.

6.3.5 Knockdowns and overexpression of the miRNAs of interest

Mesangial cells were infected with lentiviruses (ABM; Table 6.4), at a multiplicity of infection of 1, and in the presence of polybrene®(3µg/mL). The lentiviruses expressed miRNA inhibitors complementary to the targeted mature miRNA sequence under the control of the H1 promoter. Green fluorescent protein and the puromycin resistance gene were under the control of the cytomegalovirus (CMV) promoter. The infection efficiency was evaluated by fluorescence with the Axio Observer A1 microscope (Carl Zeiss). Stable knockdowns (KD) were obtained with puromycin selection and were used as polyclonal populations. The controls were mesangial cells transduced with a lentivirus vector and uninfected cells.

Name	Company	Catalog Number	Code
hsa-miR-26a	abm	mh35378	LentimiRa-Off-hsa-miR26a
hsa-miR-30b	abm	mh35422	LentimiRa-Off-hsa-miR30b
hsa-miR-4286	abm	mh35785	LentimiRa-Off-hsa-miR4286
Control lentivirus	abm	m008	Lenti-III-miR off Control Virus

Table 6.4 – Lentiviruses used for the knock-downs of the miRNAs of interest.

RNA was extracted with Qiagen RNAeasy kit and analyzed with 2100 Bioanalyzer (Agilent). The transcriptome was amplified with Ovation Pico WTA System V2 (Nugen). Whole genome expression of the KDs and controls was studied using Affymetrix GeneChip Human Gene 2.0 ST arrays. Gene expression data were normalized and quality controls were assessed before further analyses. Canonical pathways were studied using Ingenuity Pathway Analysis (IPA) and selecting the top 250 genes whose expression was increased in the KD when compared to controls.

The Clontech Advantage RT kit was used to generate complementary DNA for the study of transcripts. Gene expression was detected by qRT-PCR and normalized to 18S ribosomal RNA. Commercially available primers were purchased from Applied Biosystems (Table 6.5).

Name	Company	Code	Description
ACTIN	Applied Biosystems	4331182	Hs99999903_m1
CCNE2	Applied Biosystems	4448892	Hs00180319_m1
E2F8	Applied Biosystems	4448892	Hs01079645_g1
FADD	Applied Biosystems	4448892	Hs04187499_m1
IL32	Applied Biosystems	4448892	Hs00992441_m1
IL7R	Applied Biosystems	4448892	Hs00902338_g1
IRF1	Applied Biosystems	4448892	Hs00971960_m1
MAD2L1	Applied Biosystems	4448892	Hs00277143_m1
MCP-1	Applied Biosystems	4331182	Hs00234140_m1
MYBL1	Applied Biosystems	4448892	Hs00277143_m1
PARP2	Applied Biosystems	4448892	Hs01003785_g1
POLQ	Applied Biosystems	4448892	Hs00981375_m1
PTGR2	Applied Biosystems	4448892	Hs01584044_m1
VCAM	Applied Biosystems	4331182	Hs01003372_m1

Table 6.5 – **Primers used for mRNA quantification.**

Overexpression of miRNAs was achieved by transfection of p-MiR constructs for miR-26a and miR-30b (OriGene).These cells were maintained in 20% calf serum. Gene expression was evaluated by qRT-PCR, as previously described.

Cell proliferation was analyzed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The cells were seeded at a density of 100,000 cells/well, cultured for three days and treated according to the manufacturer's protocol (Sigma). The number of viable cells was calculated according to a standard curve. As a validation strategy, cell proliferation was also measured after six days of culture, using propidium iodide (10 mg/ml; Sigma) staining with Igepal permeabilization followed by spectrophotometric detection.

6.3.6 Statistical analysis

GraphPad Prism software version 5.0 was used for the statistical analysis. Unpaired t tests and Mann-Whitney U tests were used for comparisons between samples with normal and non-normal distributions, respectively. P values <0.05 were considered statistically significant. *In vitro* experiments were performed at least three times.

6.4 RESULTS

6.4.1 Lupus nephritis has a characteristic kidney miRNA signature that reflects cell proliferation

The expression of more than 700 miRNAs was analyzed in the kidneys of controls and children with lupus nephritis and post-streptococcal glomerulonephritis using a high-throughput methodology. The principal component analysis showed that broadly the three groups clustered in different parts of the diagram (Figure 6.1). No differences were seen between the different types of controls. These findings suggested that the renal miRNA pattern seen in lupus nephritis patients is characteristic of the disease and reflects its specific pathogenesis.

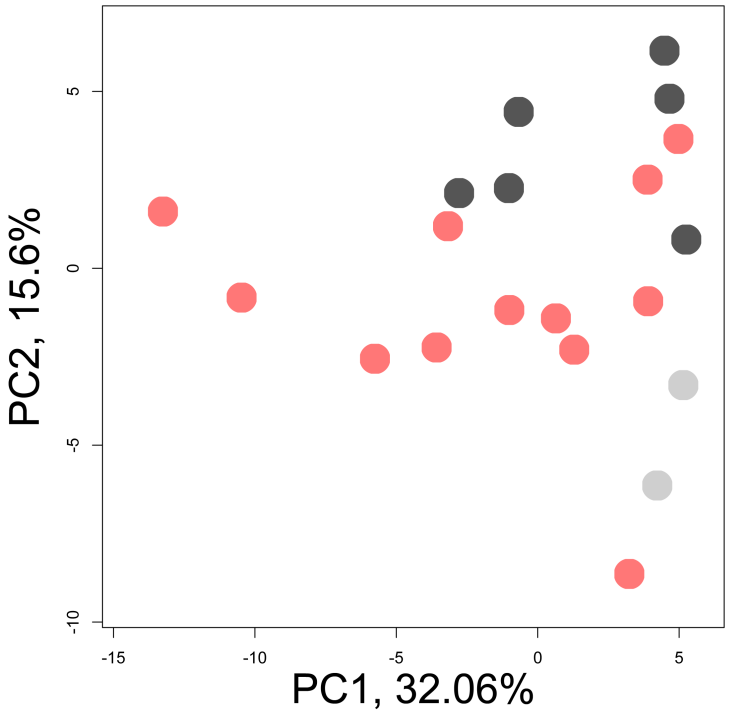


Figure 6.1 – Principal component analysis using the data of all measured kidney miRNAs. The unsupervised clustering of samples showed that healthy controls (N=6; black dots), children with lupus nephritis (N=12; red dots) and children with post-streptococcal glomerulonephritis (N=2; grey dots) had distinctive patterns of miRNA expression. The top two principal components accounted for about half of the total variance and their plot suggested that the three sample groups had different miRNA signatures. The lupus nephritis samples clustered together on the diagram, while the normal samples and the post-streptococcal glomerulonephritis samples were further apart, also forming clusters. The normal and post-streptococcal glomerulonephritis groups both formed relatively smaller clusters, so samples within the same group had smaller difference in terms of miRNA expression. The lupus nephritis group formed its own cluster, but with larger sample-sample difference, suggesting that, while in general lupus nephritis samples changed their miRNA expression, some samples had more dramatic changes than others.

The miRNAs that were significantly altered in the kidneys of lupus nephritis patients when compared to controls are displayed in Table 6.6 and in Table 6.7.

Levels of 41 miRNAs were significantly decreased in the kidneys of lupus nephritis patients when compared to controls ($p<0.05$; Table 6.6). According to IPA, the miRNAs decreased in lupus nephritis were associated with cell cycle (p= 8.21E-05 - 2.11E-02).

We selected 3 miRNAs for further study (miR-26a, miR-30b, and miR-4286) (Figure 6.2A) based on the magnitude of their differential expression and p values ($p< 0.0001$, $p= 0.0045$, and $p< 0.0001$, respectively). There were no significant differences in the expression of these miRNAs according to sex, ethnicity, age, or immunosuppressive regimen used. Patients without previous treatment also had a statistically significant decrease in miR-26a, miR-30b, and miR-4286 compared to controls ($p= 0.0005$, $p= 0.0244$, and $p= 0.0003$, respectively). Levels of housekeeping genes were no different between lupus nephritis patients and controls. The data for miR-26a and miR-30b levels were validated by qRT-PCR.

We next focused our functional analyses on miR-26a and miR-30b, since miR-4286 was recently described and its cellular functions are still unknown.

The fragmented RNA from the paraffin-embedded samples could not be interrogated to confirm a transcriptome signature related to dysregulation of miR-26a and miR-30b; however, we performed an *in silico* analysis of data available publicly (17). The transcriptome in lupus nephritis glomeruli showed a significant de-repression of predicted miR-26a and miR-30b targets (Figure 6.3).

DAVID terms enriched in the miR-26a targets up-regulated in lupus nephritis glomeruli were blood pressure, transmembrane proteins, defense response, response to wounding, immune response, and lipoproteins ($p<10^{-4}$). For miR-30b, the terms were immune response, disulfide bond, regulation of blood pressure, and regulation of proliferation ($p<10^{-4}$). These data support the concept that miR-26a and miR-30b dysregulation is biologically relevant in lupus nephritis.

Gene Name	T test Normal vs. Lupus Nephritis	Fold difference Lupus Nephritis/Normal
hsa-miR-4286	2.12853E-05	0.089536526
hsa-miR-26a	4.36969E-05	0.423574285
hsa-miR-23b	0.000177406	0.633675638
hsa-miR-30c	0.000222236	0.363989142
hsa-let-7e	0.000737622	0.661720546
hsa-miR-663a	0.00359616	0.426168113
hsa-miR-1973	0.003630868	0.339647702
hsa-miR-30b	0.004535288	0.434049392
hsa-let-7a	0.004561134	0.618014422
hsa-miR-145	0.00495462	0.582368133
hsa-miR-423-5p	0.005203153	0.592909576
hsa-let-7b	0.00584016	0.685095507
hsa-miR-637	0.006001927	0.619592875
hsa-miR-30e	0.007082057	0.640052785
hsa-miR-626	0.009620165	0.554575524
hsa-let-7c	0.013890988	0.649720169
hsa-miR-604	0.013976949	0.675510401
hsa-miR-876-3p	0.01427286	0.572573562
hsa-miR-596	0.014412144	0.540657199
hsa-miR-339-5p	0.015042612	0.634662327
hsa-let-7f	0.015236723	0.47854804
hsa-miR-29c	0.01829084	0.689901074
hsa-miR-630	0.018796748	0.221340836
hsa-miR-30a	0.019323611	0.713582478
hsa-miR-20a+hsa-miR-20b	0.022759215	0.66281194
hsa-miR-98	0.024476095	0.709887433
hsa-miR-4284	0.024531451	0.567944133
hsa-miR-572	0.026212829	0.667400419
hsa-miR-22	0.030640897	0.698466309
hsa-miR-210	0.031714249	0.450475436
hsa-miR-525-5p	0.033482294	0.687291952
hsa-miR-1298	0.037455011	0.581829496
hsa-miR-1269a	0.043284428	0.675144988
hsa-miR-578	0.043965959	0.77804043
hsa-miR-517c-3p+hsa-miR-519a-3p	0.046464016	0.678564482
hsa-miR-422a	0.048339795	0.770921214
hsa-miR-193b-3p	0.048588633	0.614699517
hsa-miR-221	0.048655838	0.551131207
hsa-miR-1243	0.049758824	0.710537105

Table 6.6 - MiRNAs decreased in the kidneys of patients with lupus nephritis class IV when compared to normal kidneys.

Gene Name	T test Normal vs. Lupus Nephritis	Fold difference Lupus Nephritis/Normal
hsa-miR-577	0.002966376	1.742928975
hsa-miR-378h	0.003441046	1.679714197
hsa-miR-1273e	0.003543853	1.775357056
hsa-miR-1827	0.003841936	2.119547757
hsa-miR-548ae	0.013253079	1.748176957
hsa-miR-576-5p	0.015078496	1.87795421
hsa-miR-548j	0.019630894	2.124509804
hsa-miR-758	0.019903329	1.601457399
hsa-miR-888-5p	0.023249248	1.831257881
hsa-miR-548ac	0.026315585	2.536134088
hsa-miR-921	0.027744692	1.932698413
hsa-miR-548d-3p	0.030733223	2.659273151
hsa-miR-603	0.03107462	2.165185916
hsa-miR-570-3p	0.032523902	1.42959854
hsa-miR-1273a	0.035392959	1.639341741
hsa-miR-548b-5p	0.039407225	1.874268191
hsa-miR-154-5p	0.04336021	1.8154
hsa-miR-574-5p	0.044504049	3.471392823
hsa-miR-302d-3p	0.044835518	1.463923036
hsa-miR-548z	0.045544296	2.282964986
hsa-miR-1264	0.046666603	1.601000196

Table 6.7 - MiRNAs increased in the kidneys of patients with lupus nephritis class IV when compared to normal kidneys.

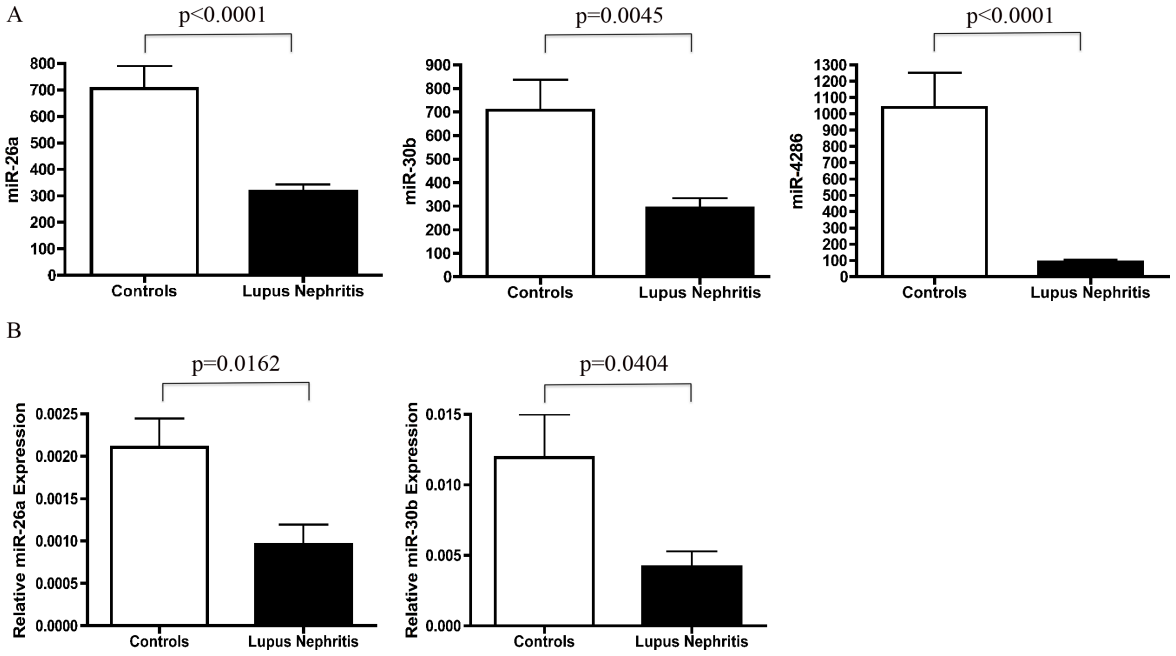


Figure 6.2 – miR-26a and miR-30b are decreased in the kidneys and urine of patients with lupus nephritis. A. Levels of miR-26a, miR-30b and miR-4286 in the kidneys of pediatric lupus nephritis patients (N=12) and controls (N=6) were measured by direct digital detection of molecular barcodes. **B.** miR-26a and miR-30b levels in the urine of adult lupus nephritis patients (N=16) and controls (N=19) were analyzed by qRT-PCR. Error bars indicate the standard error of the mean.

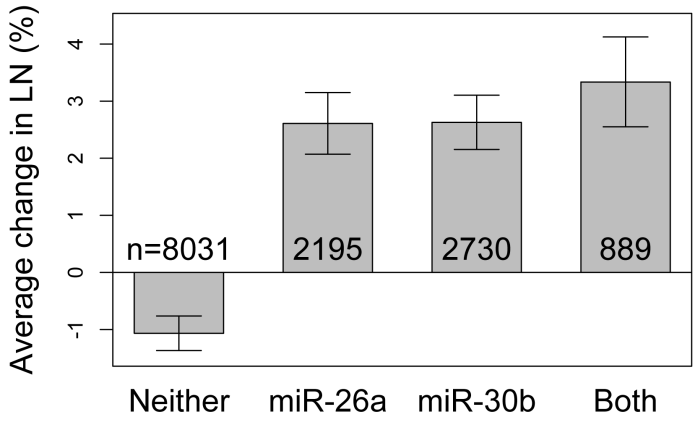


Figure 6.3 – Analysis of the lupus nephritis glomerular transcriptome using an *in silico* approach. Predicted target genes of 249 miRNAs were downloaded from www.microrna.org (August 2010 release). The raw data of GSE32591 were downloaded from GEO database and processed using the RMA method and custom library file provided by the BRAINARRAY project. This database includes results from Affymetrix microarrays performed with RNA from glomeruli of 32 lupus nephritis patients and 15 healthy kidney donors. The processed data includes 12,067 unique NCBI genes. The differential expression of each gene between lupus nephritis and healthy kidneys was calculated as the log2-ratio of group means. Targets were mapped to the GSE32591 data set to get those included in the data set. Lupus nephritis-healthy kidneys log2-ratios of the targets of the same miRNA were averaged and converted to percentage change. Statistical significance of log2-ratio values between targets and non-targets of each miRNA was obtained by Student's t test. We found that miR-26a and miR-30b predicted targets were significantly increased in the glomeruli of lupus nephritis patients when compared to controls, consistent with our findings of decreased levels of miR-26a and miR-30b in lupus nephritis. The error bars are standard deviation.

6.4.2 Urinary miR-26a and miR-30b levels are decreased in lupus nephritis

We analyzed the urinary levels of miR-26a and miR-30b to explore their potential role as lupus nephritis biomarkers. We also observed decreased levels of these miRNAs in the urine of adults with lupus nephritis when compared to healthy controls (p=0.0162; p=0.0404, respectively; Figure 6.2B), as was the case in renal tissues.

6.4.3 Mesangial cells with Knock downs of miR-26a, miR-30b or miR-4286 have a higher expression of genes related to cell cycle and DNA replication

Cell cycle pathways were strongly enriched in our analysis of the lupus nephritis miRNA signature and proliferation is a hallmark of lupus nephritis. We, therefore, investigated whether these miRNAs directly regulated cell cycle transcript abundance. We knocked down each implicated miRNA in human mesangial cells and analyzed the gene expression by arrays. According to IPA, the KD of miR-26a led to increased expression of genes associated with cell cycle (p=9.98E-09 – 1.98E-02) and DNA replication, recombination and repair (p=1.10E-08 – 1.98E-02). Similar results were obtained for miR-30b KD (p=1.47E-20 – 1.11E-02 for both types of genes) and miR-4286 KD (p=9.39E-22 – 1.15E-02 for both types of genes). These findings suggest that miR-26a, miR-30b and miR-4286 are directly regulating the expression of genes involved in cell proliferation. The expression levels of 10 genes were validated by qRT-PCR (Figure 6.4), confirming that genes associated with the cell cycle such as *CCNE2*, *E2F8*, *MAD2L1*, *MYBL1* and *POLQ*, were increased in at least one of the miRNA KD when compared to mesangial cells transduced with the lentivirus control.

Conversely, when miR-26a and miR-30b overexpression vectors were transfected into mesangial cells, we saw decreased expression of the cell cycle genes (Figure 6.5). Thus, the miRNA signature appears to be intimately involved in pathologic gene expression in lupus nephritis.

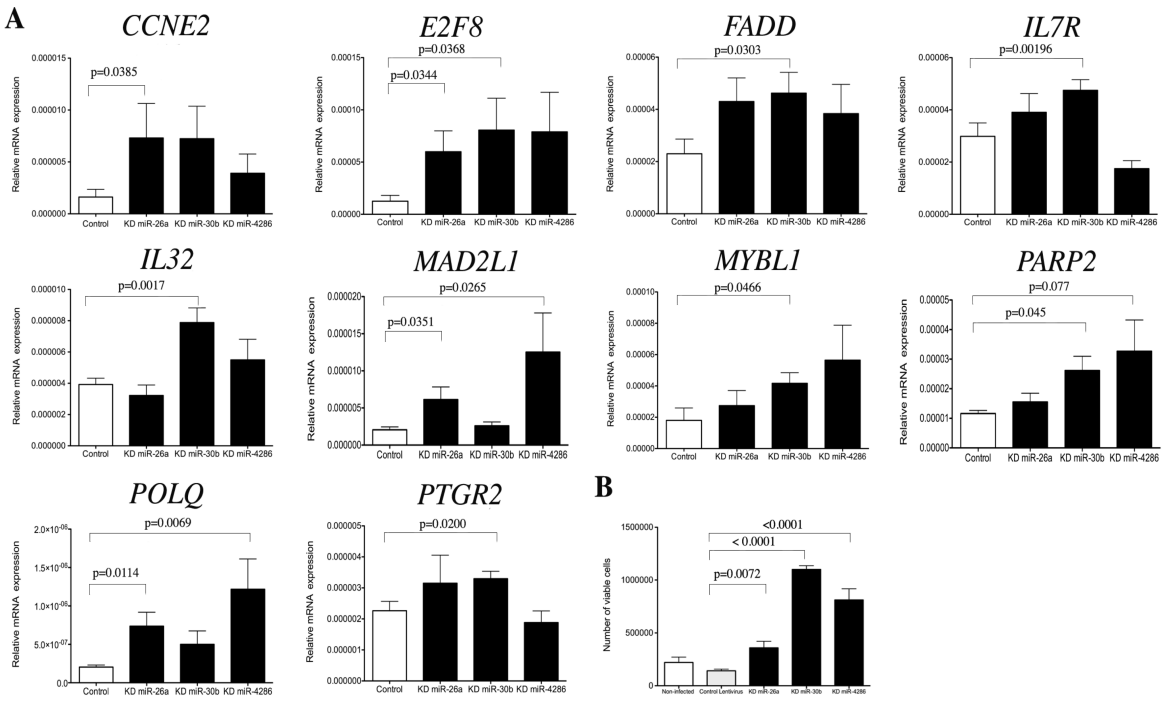


Figure 6.4 - Mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286 have higher expression of genes related to cell cycle and proliferate more. **A.** Expression of different miRNA targets in mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286 and in vector-transduced control cells was evaluated by qRT-PCR. **B.** MTT assays were performed to study cell proliferation in mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286, in cells transduced with a lentivirus control vector, and in uninfected cells.

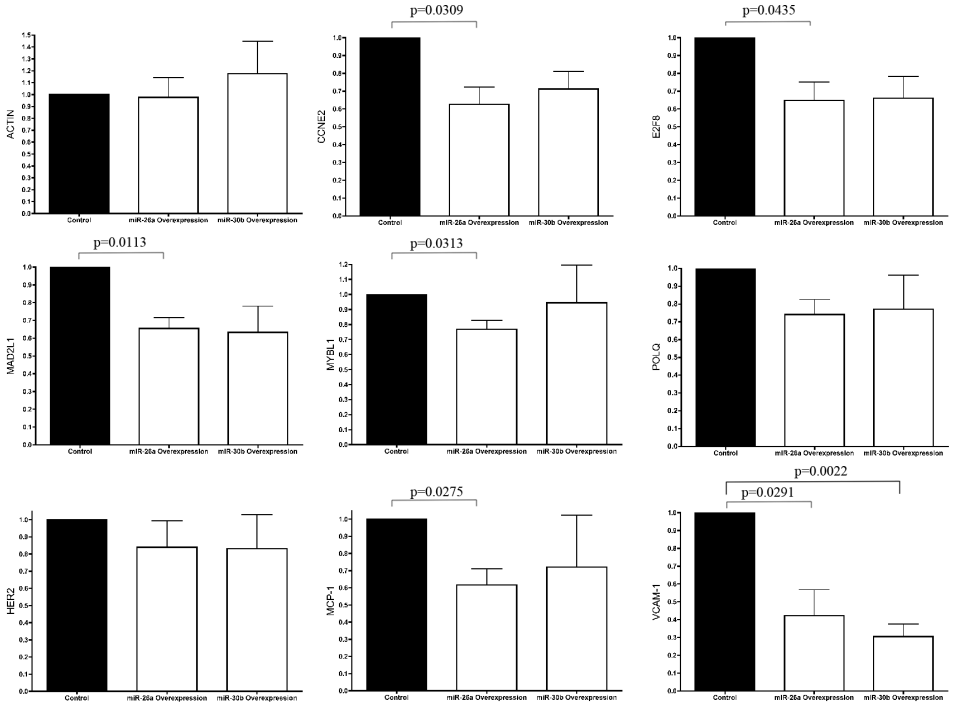


Figure 6.5 – Mesangial cells with mimics of miR-26a or miR-30b have lower expression of cell cycle related genes. Expression of different miRNA targets in mesangial cells with overexpression of miR-26a or miR-30b and in vector-control cells was evaluated by qRT-PCR. Proportions of the overexpressing cells versus the controls are presented. The experiments were performed four times.

6.4.4 Mesangial cells with miR-26a, miR-30b or miR-4286 KD proliferate more than those infected with lentivirus controls

We directly examined proliferation by performing MTT assays. A statistically significant increase in the number of viable mesangial cells was seen with knockdown of miR-26a, miR-30b, or miR-4286 compared to cells transduced with the lentivirus control ($P=0.0072$, $P<0.0001$, and $P<0.0001$, respectively) (Figure 6.4B and Figure 6.6). These data were further confirmed using propidium iodide to measure DNA content (Figure 6.7).

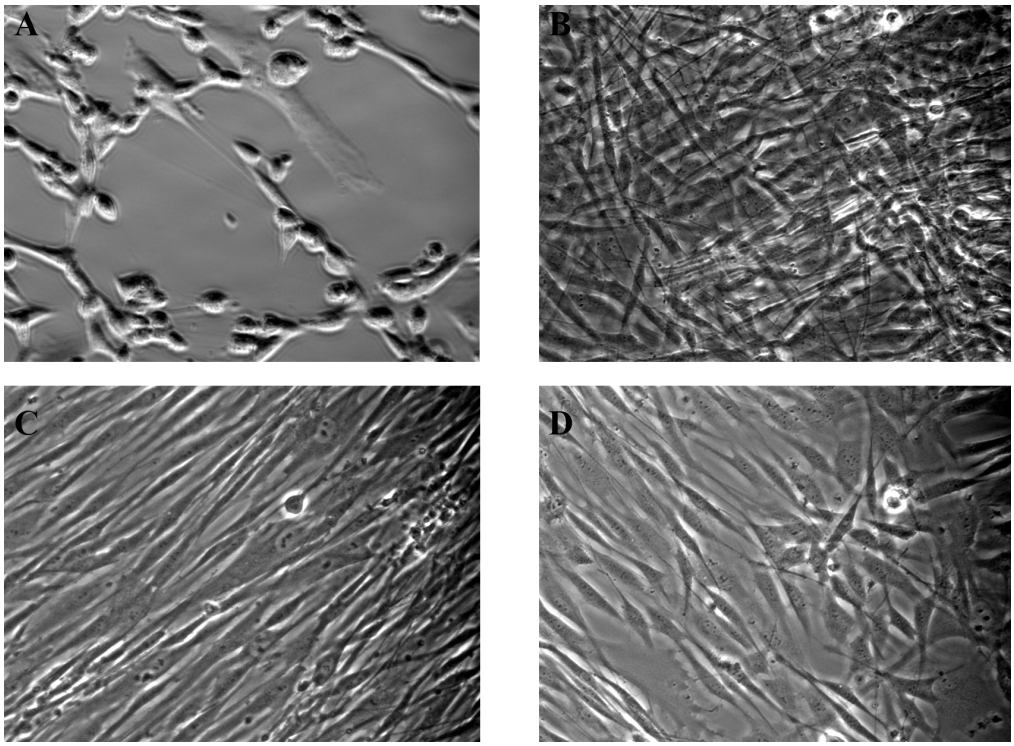


Figure 6.6 – Human mesangial cells after three days of culture. A. Lentivirus control. **B.** miR-26a knock-down. **C.** miR-30b knockdown. **D.** miR-4286 knockdown. All pictures were taken with 200x magnification.

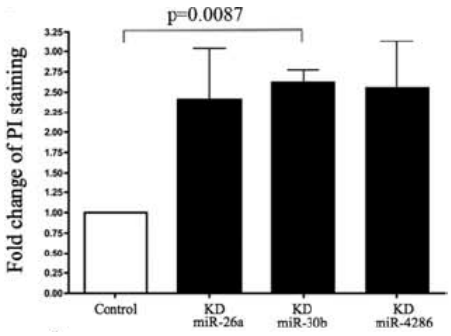


Figure 6.7 – Study of cell proliferation with propidium iodide (PI). Mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286 were cultured for 6 days and evaluated with PI.

6.4.5 MCP1 and VCAM1 levels are regulated by miRNAs

MCP1 and VCAM1 are thought to participate in the infiltration of the kidneys by inflammatory cells, a well-known process in lupus nephritis pathogenesis. Our *in silico* studies identified VCAM1 as a possible miR-30b target in lupus nephritis kidneys (Figure 6.3). We therefore examined MCP1 and VCAM1 levels in mesangial cells overexpressing miR-26a and miR-30b, and we found significantly decreased expression of both MCP1 and VCAM1 in cells overexpressing miR-26a and decreased expression of VCAM1 in cells overexpressing miR-30b (Figure 6.8).

These data suggest that proliferation and inflammation are both influenced by these miRNAs.

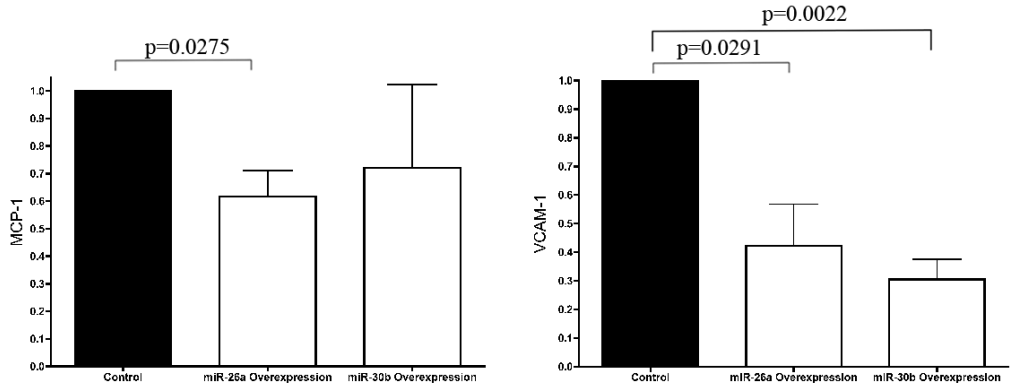


Figure 6.8 – MCP1 and VCAM1 expression is controlled by miRNAs.

6.5 DISCUSSION

Gene expression is regulated, on the post-transcriptional level, by miRNAs. A broad change in the miRNA pattern of a cell can, therefore, lead to the global dysregulation of its mechanisms and contribute to disease pathogenesis and chronicity. MiRNAs are essential for the normal function of kidney cells. Mice lacking mature miRNAs in podocytes due to podocyte-specific Dicer KO had significant proteinuria and rapid progressive glomerular and tubular damage, in association with aberrant apoptosis and increased cell proliferation (607). Furthermore, after four weeks of age, glomerular tuff collapse and crescent formation were observed in almost all of the mutant glomeruli, findings similar to those seen in severe lupus nephritis (607).

With this project, we identified a lupus nephritis miRNA signature, which, overall, reflected mainly cell proliferation. Moreover, the lupus nephritis miRNA pattern was different from the one observed in the kidneys of children with post-streptococcal glomerulonephritis, another mesangio-proliferative disease, supporting specificity.

The study of a pediatric cohort is more informative than an adult one, since lupus nephritis is particularly active in children (603,608–610) and co-morbidities, like diabetes mellitus or hypertension, are rare in this age group, as previously discussed.

Using a high throughput methodology we identified, for the first time, a significant decrease in miR-26a, miR-30b and miR-4286 in the kidneys of lupus nephritis patients.

On the MTT assays the cells submitted to the KD of miR-26a, miR-30b or miR-4286 proliferated significantly more than the ones infected with the lentivirus control. In addition, the genes found to be upregulated in the KDs of the miRNAs were associated with cell cycle and DNA replication, including *CCNE2* and *E2F8*, which play a role in G_1/S transition. Notably, the effect on gene expression is significant but not dramatic, which is consistent with the notion that individual miRNAs impact cellular processes by modestly repressing the levels of several proteins.

The miRanda algorithm (www.microrna.org) predicted that seven (*CCNE2*, *E2F8*, *FADD*, *MAD2L1*, *MYBL1*, *POLQ* and *PTGR2)* out of the ten target genes identified through the array study of the KDs had possible alignment sequences with miR-26a, miR-30b or miR-4286. In some cases there were more than one possible alignment sequence and more than one of these three miRNAs involved. The case of *CCNE2* is paradigmatic, since the miRanda algorithm identified one possible sequence alignment for miR-26a and two for miR-30b. Moreover, these two miRNAs are included in the top-ten miRNAs considered to have a higher sum of the miRSVR scores for *CCNE2*, so the miRNAs with a higher likelihood to regulate the expression of this gene.

PicTar, another algorithm for the identification of miRNA targets, also predicted that CCNE2 would be a target for both miR-26a and miR-30b.

Our data showed that in the kidneys of lupus nephritis patients there is a significant decrease of miR-26a, miR-30b and miR-4286 and that these miRNAs regulate the expression of genes that participate in the cell cycle. This observation is consistent with the literature, considering that a decrease on these miRNAs has also been identified

in other diseases characterized by cell proliferation dysregulation. miR-26a was found to be down-regulated in several malignancies, including nasopharyngeal carcinoma (611), esophageal adenocarcinoma (612), pancreatic duct adenocarcinoma (613), liver cancer (614–617), breast cancer (618–620), lung cancer (621–623), thyroid cancer (624,625), prostate cancer (626–628), clear cell renal cell carcinoma (629), bladder cancer (630,631), lymphoma (632,633), rhabdomyosarcoma (634), osteosarcoma (635), cutaneous squamous cells carcinoma (636) and melanoma (637–639).

In hepatocellular carcinomas not only is miR-26a downregulated in both human and mouse tumors (614,616,617) and an independent predictor of survival (616,617,640) and response to IFNα (616), but it is also inversely correlated with NFkB and IL6 levels (616,617). Moreover, it directly down-regulates cyclins D2 and E2 (614) and induces a G_1 arrest (614,617). Ectopic expression of miR-26a suppresses tumor growth and vascularity in mice, being inversely correlated with the levels of vascular endothelial growth factor (VEGF) (640,641). Furthermore, delivery of miR-26a by adeno-associated virus was shown to reduce cancer cell progression and cause tumor-specific apoptosis *in vivo* (614), indicating that miR-26a is involved in the regulation of cell proliferation and apoptosis. Interestingly, it was also reported that down-regulation of miR-26a promotes mouse hepatocyte proliferation during liver regeneration in association, once again, with *CCNE2* overexpression (642).

Data regarding other malignancies should also be mentioned. Ectopic miR-26a was reported to inhibit cell proliferation and induce cell cycle arrest at G_1 phase in nasopharyngeal carcinoma cell lines and suppress tumorigenesis in a murine model of nasopharyngeal carcinoma cells xenograft (611). Notably, in nasopharyngeal carcinoma-derived cell lines miR-26a down-regulated the expression of *CCNE2*. This finding supports our hypothesis that CCNE2 is an mRNA target of miR-26a.

In pancreatic duct adenocarcinomas a miR-26a down-regulation was identified by qRT-PCR and *in situ* hybridization (613). Moreover, the 1- and 3-year survival rates were 40% and 0%, respectively, in the miR-26a-negative group, but were 50% and 6%, respectively, in the miR-26a-positive group. The multivariate survival analysis of conventional clinical prognostic factors showed that miR-26a is an independent prognostic factor in pancreatic cancer (613). Finally, the *in vitro* and *in vivo* assays performed showed that miR-26a overexpression resulted in G_1 arrest, cell proliferation

inhibition and a decrease in tumor growth, phenomena associated once again with *CCNE2* downregulation. Similar results were found for breast and gastric cancer (618,620,643).

The genome-wide miRNA expression profile of thyroid anaplastic carcinomas and normal thyroid tissue identified a significant decrease on miR-26a levels on the tumors (624). These results were confirmed by Northern Blot and qRT-PCR (624). Transgenic mice expressing SV40 large T oncogene under the transcriptional control of the thyroglobulin promoter developed undifferentiated thyroid carcinomas (644) and these tumors were also found to have a decrease on miR-26a expression (624). *In vitro*, the overexpression of miR-26a in two human derived thyroid anaplastic carcinoma cell lines caused cell growth inhibition (624), which supports the evidence for a role for miR-26a on the control of cell proliferation in this type of cells.

Finally, there is evidence that ectopic miR-26a also decreases cell proliferation in cell lines derived of bladder cancer (631), lymphoma (633) and acute myeloid leukemia (645). In the latter the control of cell proliferation is once again, at least in part, mediated by *CCNE2* down-regulation (645).

Interesting data connect miR-26a to the MYC pathway. Koh and collaborators reported that in the mouse prostate and, *in vitro*, in human prostate cancer cells, upregulation of MYC was associated with a decrease on miR-26a and an overexpression of *EZH2*. These data suggest, therefore, that MYC participated in prostate tumor initiation and disease progression through a mechanism, at least in part, related to miR-26a (626). It was also reported that MYC can directly bind upstream of EZH2 and activate its transcription. EZH2 is a histone lysine methyltransferase that catalyzes the tri-methylation of histone 3 on lysine 27 (H2K27me3), leading to chromatin remodeling and gene silencing. Some of the genes whose expression is controlled by EZH2 are homeobox genes involved in differentiation. The overexpression of EZH2 is, therefore, associated with the promotion of cell proliferation, invasion and metastasis formation. According to this group, the pathway MYC-miR-26a-EZH2 has an important role in prostatic cancer tumorigenesis (626).

A CHIP assay demonstrated that MYC also recruited EZH2 to the miR-26a promoters and both cooperatively repressed miR-26a expression in aggressive B-cell lymphoma cell lines (633). MYC activation and its consequent repression of miR-26a was

proven to be associated with lymphoma aggressiveness and progression. The combined treatment with both JQ1, a MYC inhibitor, and DZNep, a EZH2 inhibitor, decreased MYC activation, increased miR-26a levels and reduced lymphoma growth and clonogenicity in aggressive lymphoma cell lines and primary lymphoma samples (633).

In conclusion, taking into account the different data presented, the miR-26a gene is now generally considered a tumor suppressor gene. Recent studies have, nonetheless, shown other roles for miR-26a.

In human esophageal adenocarcinoma miR-26a expression was significantly decreased in tumor cells when compared to normal mucosa, and in liver and lymph nodes metastases when compared to the primary tumor. The analysis, by arrays, of the miRNA expression of the orthotopic transplantation of esophageal adenocarcinomas and the corresponding liver metastasis identified a group of miRNAs, which included miR-26a, that seem to be involved in the process of metastasis formation. By qRT-PCR, it was confirmed that miR-26a expression was significantly decreased in liver and lymph nodes metastases compared to primary esophageal adenocarcinoma tissues (612). Zhang YF and collaborators further tested the hypothesis of miR-26a being involved in metastasis formation, by performing *in vitro* studies with OE33 and OE33/AR cells (612). The former is a cell line of esophageal adenocarcinoma, and the latter is a subpopulation that acquired resistance to anoikis. They showed that antisense-mediated suppression of miR-26a expression in OE33 cells resulted in significant inhibition of anoikis, while increasing miR-26a levels by transfection of miR-26a mimics increased the sensitivity of OE33/AR cells to anoikis (612). It was, therefore, suggested a role for miR-26a not only in tumorigenesis of esophageal adenocarcinoma, but also in the control of anoikis-resistance and in the formation of metastasis (612).

The spectrum of functions of miR-26a is even broader, considering that in other types of cancer, like cholangiocarcinoma (646), ovarian cancer (647), liposarcoma (648) and glioma (649,650), miR-26a is clearly an oncogene, since it is overexpressed in tumor specimens and cell lines and it directly promotes cell proliferation, colony formation and tumor progression. In the case of the gliomas there is also robust evidence that miR-26a alone promotes transformation and growth and that it markedly enhances the transcription of VEGF *in vitro* and promotes tumor angiogenesis *in vivo* (650).

Finally, for certain types of malignancies the role of miR-26a remains elusive,

because different groups have presented contradictory results. This is the case of lung cancer. Dang X and collaborators showed that miR-26a expression was down-regulated in human lung cancer specimens and that the overexpression of miR-26a in the A549 human lung cancer cell line caused a dramatic inhibition of cell proliferation, G_1/S phase block, activation of apoptosis and inhibition of invasion and metastasis formation (623). Liu B *et al.* also found that miR-26a was down-regulated in lung cancer specimens and cell lines, but, unexpectedly, this group found that miR-26a was up-regulated in lymph node metastasis when compared with primary tumors (622). Moreover, it was shown in three different human lung cancer cell lines that the ectopic expression of miR-26a dramatically enhanced lung cancer cell invasion and migration (622). It was suggested that these phenomena occurred, at least in part, by decreasing PTEN expression and increasing the expression of VEGF, Twist, β-catenin and matrix metallopeptidase 2 (MMP2) (622). These proteins participate in mechanisms that are essential for tumorigenesis and metastasis formation. New data are, therefore, necessary to further clarify the real role of miR-26a in the lung cancer pathogenesis.

Apart from cancer, miR-26a dysregulation has also been associated with idiopathic pulmonary fibrosis (651,652) and autoimmune diseases, like primary biliary cirrhosis (653) and rheumatoid arthritis (654). In a rat model, miR-26a represses *TLR3* in macrophages and improves the severity of pristine induced arthritis (655).

Finally, miR-26a also seems to be important for the normal functioning of the kidneys. By microarray expression profiling and *in situ* hybridization, mmu-miR-26a was identified as having a pan-glomerular expression pattern in the kidneys of wild-type mice (607). Furthermore, another group showed that miR-26a was the most abundantly expressed miRNA in the glomerulus of normal C57BL/6 and that its glomerular expression in B6.MRLc1 mice was significantly lower (656). Moreover, glomerular miR-26a correlated negatively with the urinary albumin levels and podocyte specific gene expression (656).

In conclusion, these findings suggest that the role of miR-26a is tissue and time specific, so that in different cell contexts this miRNA can have different biologically relevant targets and markedly different roles, many of which can be unique or even opposite to the ones found in other cells. Nevertheless, globally miR-26a has mainly been associated with the regulation of cell proliferation.

Regarding miR-30b, there is also evidence that it is down-regulated in several malignancies, including colorectal cancer (657,658), squamous cell lung carcinoma (621) and invasive bladder carcinoma (659). Regarding colorectal cancer, it was documented that miR-30b was significantly down-regulated in primary tumor specimens (657,658) and in liver metastasis tissues (658). *In vitro* studies of miR-30b overexpression showed an inhibition of cell proliferation, migration and invasion (657,658), with induction of G_1 arrest and apoptosis (657). A reduction of tumor growth was reported in a nude mice xenograft model (657). In the case of both colorectal cancer and invasive bladder carcinoma miR-30b was found to be a predictor for survival (657–659).

Both miR-30b and miR-26a were decreased in aggressive primary metastatic clear cell renal cell carcinomas (629).

Decreased levels of miR-26a and miR-30b were also found in the sera of patients with systemic sclerosis, being associated with fibrosis (660). The down-regulation of miR-30b was reported in the skin of these patients and in mice with bleomycin-induced dermal fibrosis (660). *In vitro* studies showed that TGFβ down-regulated miR-30b expression in dermal fibroblasts, which caused an increase on PDGFRβ expression and, subsequently, collagen production. Transfection of dermal fibroblasts with a miR-30b mimic repressed PDGFRβ expression, inhibited collagen synthesis and impaired the differentiation of dermal fibroblasts into myofibroblasts (660).

In conclusion, there is robust evidence in the literature supporting the roles of miR-26a and miR-30b as key regulatory molecules for the control of cell proliferation and apoptosis. Moreover, new data show that these miRNAs may also inhibit pro-fibrotic signals. In our study we found a significant decrease of these miRNAs in lupus nephritis, a disease whose pathogenesis is characterized precisely by cell proliferation and fibrosis.

The stability of miRNAs in urine and the feasibility of obtaining such samples make these regulatory molecules promising candidates as non-invasive biomarkers for several diseases. In this study we showed that not only miR-26 and miR-30b are significantly decreased in the kidneys of lupus nephritis patients, but also in the urine of these patients. Further studies are necessary to define the true value of these molecules in clinical practice comparing to other well-known lupus nephritis biomarkers.

With the improvement of short RNA delivery methods, miRNA replacement therapies with mimics or suppression of oncogenic miRNAs with miRNA inhibitors have

been suggested as potential therapeutic approaches for cancer and we could extrapolate that these would also be a possibility in lupus nephritis. Nevertheless, the utility of therapies that target specific miRNAs should be carefully addressed, considering that a single miRNA regulates the expression of multiple genes and may affect several mechanisms in different cells. Kota and collaborators hypothesized that the most therapeutically useful miRNAs will be the ones expressed in low levels in tumors and highly expressed and, therefore, tolerated, in normal tissues. This group showed that adenovirus mediated miR-26a delivery suppressed cancer cell proliferation and activated tumor-specific apoptosis in a hepatocarcinoma mouse model, without causing toxicity (614). Since miR-26a is highly expressed in the normal kidneys and significantly decreased in lupus nephritis, we also postulate that this miRNA might have a therapeutic interest. miRNA delivery would thus allow the therapeutic restitution of the physiologic program of the cells, which was lost in lupus nephritis.

6.6 CONCLUSIONS

We identified, for the first time, the kidney lupus nephritis characteristic miRNA signature. Furthermore, we showed that miR-26a and miR-30b are significantly decreased in the kidneys and in the urine of these patients and that these miRNAs are responsible for the control of cell proliferation. A new pathway in lupus nephritis pathophysiology was, therefore, described, which opens the door for further studies on the practical role of miR-26 and miR-30b as biomarkers in lupus nephritis.

Please refer to **Appendix A.3** for the paper ***The role of MicroRNAs and Human Epidermal Growth Receptor 2 in Proliferative Lupus Nephritis***, which was published in the journal *Arthritis and Rheumatology*.

CHAPTER 7

HER2: A New Player in the Pathogenesis of Lupus Nephritis

7.1 INTRODUCTION

The underlying mechanisms responsible for the dysregulation of the miRNA pattern of a cell are not well-understood yet, but several have been suggested, including transcriptional inhibition, epigenetic modifications, DNA copy number abnormalities and defects in the miRNA machinery.

As further discussed in Chapter 6, in prostatic cancer cell lines MYC decreased miR-26a expression by direct binding at the promoter regions of *CTDSPL* and *CTDSP2*, genes whose introns harbor the miR-26a primary transcripts (626). In a murine lymphoma model with the *MYC* transgene expression controlled in a Tet-off system, the miRNA expression profiling, performed with microarrays, also showed that MYC repressed miR-26a (661). Moreover, a CHIP assay demonstrated that MYC recruited EZH2 to a miR-26a promoter and cooperatively repressed miR-26a expression in lymphoma cell lines (633). Other mechanisms of miR-26a control have also been described. It was shown, for example, in prostate cancer cell lines that miR-26a suppression was associated with the hypermethylation of the miR-26a locus (627).

In breast cancer cells, it was found that trastuzumab, a monoclonal antibody against the Human Epidermal Growth Factor Receptor 2 (HER2), caused a G_1 arrest by up-regulating miR-26a and miR-30b (619). Since we identified a significant decrease in these two miRNAs in the kidneys of patients with lupus nephritis, we hypothesized that HER2 might have a role in its pathogenesis.

7.2 GOAL

Our goal was to characterize the role of HER2 in lupus nephritis.

7.3 METHODS

7.3.1 Effect of an anti-HER2 drug in human mesangial cells

Mesangial cells were exposed to 8µg/mL trastuzumab. After 24h of culture miR-26a and miR-30b were quantified by qRT-PCR, using *C. elegans* miR-238 and U6 as controls.

After three days of culture, RNA was extracted and whole-genome expression arrays were performed. The results were compared to mock-treated mesangial cells.

7.3.2 Immunohistochemistry for HER2 in Humans

HER2 expression was analyzed by immunohistochemistry. HER2-positive breast cancer tissue was used as a positive control. For every sample, negative staining controls were executed without the use of primary antibody. Fully automated immunohistochemistry was performed on a Leica Bond-Max, with rabbit anti-HER2 (1:500; 1 hour staining; Sigma-Aldrich), after antigen retrieval at low-pH for 20 min. The Bond polymer refine detection system was used, as described by the manufacturers (Leica Biosystems). The sections were counterstained with hematoxylin. Images were obtained using Aperio ScanScope eSlide capture device.

7.3.3 HER2 immunohistochemistry in a lupus nephritis mouse model

HER2 expression was studied in NZM2410 mice (662), a lupus nephritis mouse model, and Balb/c and C57BL/6 (B6) mice, non-autoimmune controls. All three strains were originally obtained from the Jackson Laboratories. Blood and urine were collected every other week from NZM2410 mice. The characteristics of the mice are presented in Table 7.1.

Features	NZM2410	Balb/c	B6
Total Number	9	3	10
Sex			
Female	4 (44%)	3 (100%)	6 (60%)
Male	5 (56%)	0 (0%)	4 (40%)
Age at Sacrifice (months)	6 ± 3	8 ± 1	4 ± 4

Table 7.1 – Characteristics of the mice studied.

The kidneys were collected and fixed in buffered formalin. Immunohistochemistry was performed with rabbit anti-HER2 (1:300; 1 hour staining; Sigma), after heat antigen retrieval with a low-pH citrate solution (Vector Labs). Amplification with Vectastain avidin/ biotinylated enzyme complex and Vector biotinylated anti-rabbit antibody was performed. Negative controls, without primary antibody, were performed in parallel. The

number of HER2-positive cells in the glomeruli of NZM2410 mice was compared to controls (Balb/c and B6 mice). On average, 113 glomeruli were analyzed per mouse. A blood urea nitrogen (BUN) higher than 30mg/dL was considered elevated. All the experiments were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia, which is an American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facility.

7.3.4 Expression studies *in vitro*

Mesangial cells were treated with IFNα (Biomedical Laboratories) for three days (100 U/mL) and HER2 expression was evaluated by qRT-PCR and compared to the one presented by mock-treated cells (primer Hs01001580_m1; Applied Biosystems).

IRF1-puromycin plasmids and vector-only plasmids were transfected into mesangial cells, using the Amaxa Cell Line Nucleofector Kit V (Lonza). IRF1 transfection was confirmed by qRT-PCR and also by immunofluorescence, using as primary antibody mouse anti-human IRF1 (1:50; 1h incubation; Santa Cruz) and as secondary antibody Alexa Fluor 546 goat anti-mouse IgG (Invitrogen). The corrected total cell fluorescence was calculated using Image J (NIH). HER2 expression was evaluated by qRT-PCR. MiR-26a and miR-30b were also measured by qRT-PCR, using spiked *C. elegans* miRNA-39 as internal control (Qiagen).

7.3.5 Quantification of HER2, MCP1 and VCAM1 in urine

Enzyme-linked immunosorbent assays (ELISA) for HER2 *(Abcam),* M*CP1* (R&D Systems) and VCAM1 (R&D Systems) were performed, according to the manufacturers' instructions, using human urine samples. Spectrophotometry was performed with EL808 (Biotek).

Urine samples from lupus nephritis patients were obtained from the Johns Hopkins lupus cohort, a prospective longitudinal study of lupus activity and outcomes (663). Samples from patients with active lupus nephritis at time of urine collection (renal SLEDAI ≥ 4) were compared with samples from adult, healthy, gender and age matched, individuals. The characteristics of the patients are available in Table 7.2.

Characteristics	Number (%) or Mean ± S.D.
Gender	
Female	40 (85%)
Male	7 (15%)
Ethnicity	
African-American	28 (60%)
Caucasian	12 (26%)
Asian	3 (6%)
Other	4 (9%)
Family history of SLE	15 (32%)
Age at SLE diagnosis (years)	23 ± 10
Age at lupus nephritis diagnosis (years)	26 ± 11
Age at time of urine collection (years)	36 ± 10
Time between diagnosis and urine collection (years)	10 ± 8
Clinical and laboratory manifestations of SLE	
Malar rash	25 (53%)
Discoid rash	15 (32%)
Photosensitivity	15 (32%)
Oral or nasal ulcers	12 (26%)
Arthritis	32 (68%)
Pleuritis	20 (43%)
Pericarditis	14 (30%)
Renal Disorder	47 (100%)
Neurologic Disorder	15 (32%)
Hematologic Disorder	34 (72%)
Hemolytic anemia	9 (19%)
Leukopenia	25 (53%)
Lymphocytopenia	20 (43%)
Thrombocytopenia	10 (21%)
Positive anti-nuclear antibody	45 (96%)
Immunologic Disorder	45 (96%)
Alopecia due to SLE	20 (43%)
Raynaud's phenomenon	18 (38%)
Cutaneous vasculitis	5 (11%)
Antibody Profile	
Anti-dsDNA	40 (85%)
Anti-Sm	24 (51%)
Anti-RNP	21 (45%)
Anti-SSA	25 (54%)*
Anti-SSB	11 (24%)*
Anti-phospholipids antibodies	31 (66%)
SELENA-SLEDAI at time of urine collection	8 ± 4
Renal SELENA-SLEDAI at time of urine collection	6 ± 3
Patients with active lupus nephritis at time of urine collection	47 (100%)
Kidney biopsy performed in a consecutive year from the urine collection	19 (40%)
Lupus nephritis class III	9 (47%)
Lupus nephritis class IV	6 (32%)
Lupus nephritis class V	4 (21%)
Treatment at time of urine collection	40 (85%)
Hydroxychloroquine	33 (70%)
Prednisone	31 (66%)
Mycophenolate mofetil	1 (2%)
Azathioprine	2 (4%)
Cyclophosphamide	0 (0%)
Rituximab	0 (0%)
Methotrexate	4 (9%)

* These antibodies were not performed in all the patients.

Table 7.2 – Characteristics of the cohort used to study HER2 in the urine.

Samples from patients with active lupus nephritis were also compared with samples from the same patients collected at least three months before the identified flare, when the renal SLEDAI was zero.

To assess the association between HER2 and biopsy results, we studied samples that were collected from patients who had a kidney biopsy within a year from the date of the urine collection.

The IRB offices of the Children's Hospital of Philadelphia and of the Johns Hopkins University School of Medicine approved this study.

Urine samples were collected into sterile containers and a protease inhibitor was added (cOmplete Protease Inhibitor Cocktail, Roche). The samples were placed on ice or refrigerated at 4°C within 1h of collection and stored at -80°C. HER2, MCP1 and VCAM1 values were normalized by dividing by the urine creatinine concentration.

7.3.6 Statistical analysis

GraphPad Prism 5.0. was used for the statistical analysis. Unpaired t tests and Mann-Whitney U tests were used for comparisons between samples. The relationship between HER2 and the other urinary biomarkers was defined by Pearson's correlation and linear regression. P values <0.05 were considered statistically significant. *In vitro* experiments were performed at least three times.

7.4 RESULTS

7.4.1 Trastuzumab increases miR-26a and miR-30b levels and decreases the expression of cell cycle related genes

Trastuzumab, a monoclonal antibody against HER2, causes a G_1 phase arrest in breast cancer cells by increasing miR-26a and miR-30b (619). We hypothesized that trastuzumab might also increase miR-26a and miR-30b in mesangial cells and affect their cell cycle. Levels of miR-26a and miR-30b were measured in trastuzumab-treated cells and were found to be increased, using either *C. elegans* miR-238 or U6 as controls (Figure 7.1).

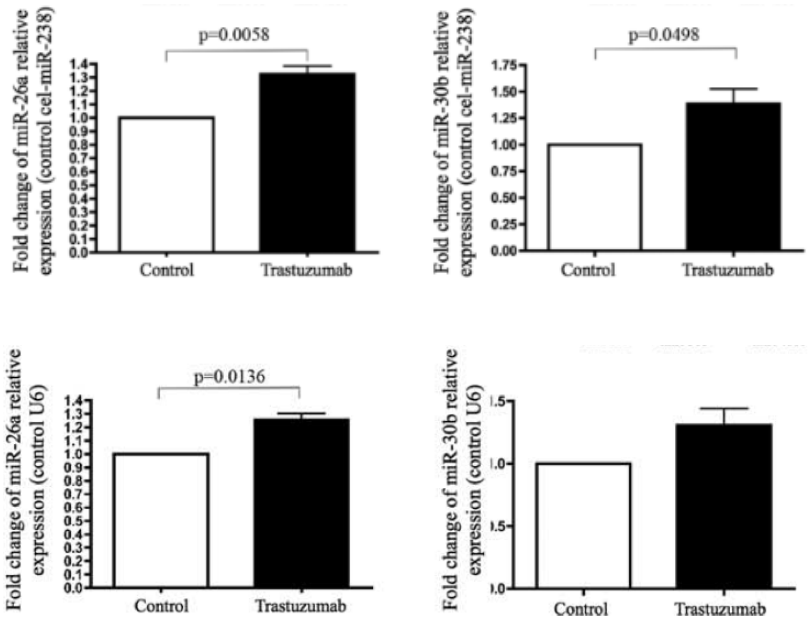


Figure 7.1 – Levels of miRNAs were measured by qRT-PCR after treatment with 8 µg/ml trastuzumab for 24 hours. Values are the mean ± SEM.

Furthermore, trastuzumab treated cells had decreased expression of genes related to cell cycle (p values of 3.96×10^{-5} to 2.70×10^{-2}) and DNA replication, recombination, and repair (p values of 1.39×10^{-8} to 2.70×10^{-2}).

These data confirm that in mesangial cells, similar to breast cancer cells, trastuzumab can increase miR-26a and miR-30b and inhibit the expression of cell cycle - related genes.

7.4.2 HER2 is dramatically increased in lupus nephritis, but not in other proliferative glomerulonephritides

The effects of trastuzumab led us to hypothesize that increased HER2 might be regulating miRNA expression in lupus nephritis. No prior studies of HER2 expression had been performed in this disease.

A dramatic increase in HER2 expression was observed in the kidneys of lupus nephritis patients, not only in the tubular compartment, but also in the glomeruli, where mesangial cells, endothelial cells and podocytes were strongly stained (Figure 7.2).

Normal kidneys and kidneys from patients with other proliferative glomerulonephritides (IgA nephropathy, poststreptococcal glomerulonephritis, and granulomatosis with polyangiitis) had light staining of tubules, but no strongly HER-2–positive cells in the glomeruli (Figure 7.2).

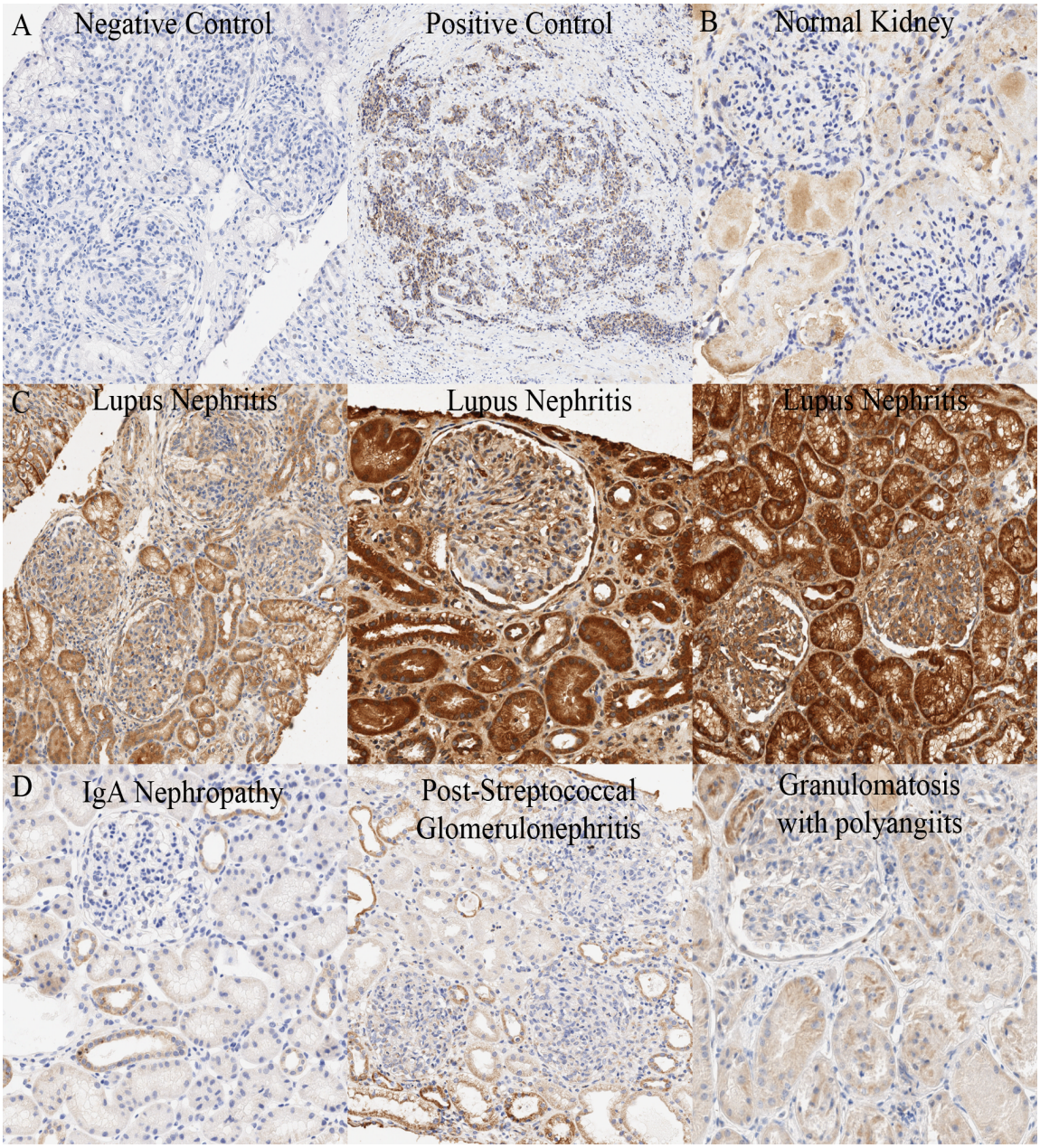


Figure 7.2 - HER2 expression was increased in the kidneys of lupus nephritis patients (N=8) when compared to normal kidneys (N=2) and other proliferative glomerulonephritides (N=12). **A.** Negative controls utilized just the secondary antibody on the kidneys of lupus nephritis patients. Positive controls were performed on $HER2^{+}$ breast cancer tissue. **B.** Kidneys from healthy donors (N=2). **C.** Kidneys from lupus nephritis patients (N=8). **D.** Kidneys from patients with other proliferative glomerulonephritides: IgA Nephropathy (4), post-streptococcal glomerulonephritis (4) and granulomatosis with polyangiitis (4). Representative samples are shown in each case.

7.4.3 The number of HER2 positive cells is increased in the glomeruli of NZM2410 mice and is associated with higher proteinuria and higher BUN levels

To further examine HER2 in lupus nephritis, immunohistochemistry studies were also conducted in NZM2410 mice and the control B6 and Balb/c mice (Figure 7.3). The NZM2410 mice developed, as expected, an early onset, aggressive, lupus-like diffuse proliferative glomerulonephritis. While in healthy humans HER2 staining was almost absent in the kidneys, in healthy B6 and Balb/c mice the tubules showed strong HER2 staining, while the glomeruli were mostly negative (Figure 7.3B). Kidneys from NZM2410 mice, however, showed a significantly higher number of $HER2^+$ cells per glomerulus when compared to B6 and Balb/c mice ($p<0.0001$ in both cases) (Figure 7.3C). In addition, the number of $HER2^+$ cells per glomerulus was significantly higher in NZM2410 mice with higher proteinuria and higher levels of BUN (Figure 7.3D), suggesting that HER2 expression correlates with the severity of the disease.

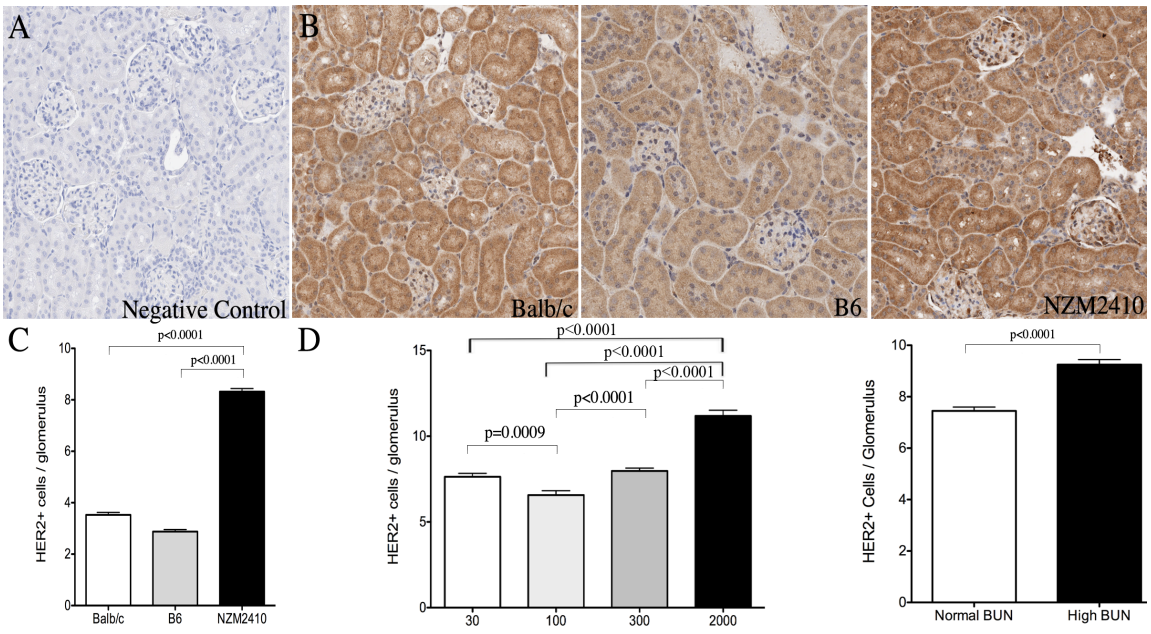


Figure 7.3 – The number of HER2 positive cells detected per glomerulus in NZM2410 (N=9) mice was significantly higher than in Balb/c (N=3) or B6 mice (N=10). **A**. A negative control performed without the use of the primary antibody on NZM2410 mouse kidneys. **B.** HER2 immunohistochemistry on the kidneys of B6, Balb/c mice and NZM2410 mice. **C.** The number of HER2 positive cells per glomerulus in NZM2410, B6 and Balb/c mice. **D.** The number of HER2 positive cells in the glomeruli of NZM2410, according to proteinuria (mg/dL) and BUN at time of sacrifice (lower or higher than 30mg/dL). Error bars indicate standard error of the mean.

7.4.4 IFNα and IRF1 increase the expression of HER2 in human mesangial cells

Our data suggested a model whereby increased HER2 expression drives decreased miR-26a and miR-30b levels, allowing for de-repression of cell cycle transcripts. We then wished to understand the etiology of the increased HER2 expression. Since type I IFNs have been implicated in SLE, we analyzed whether IFNα could regulate HER2 expression.

Firstly, we examined the expression of HER2 in several types of human renal cells by immunofluorescence and we verified that human podocytes, mesangial and tubular cells expressed this protein (Figure 7.4). We decided to use mesangial cells in the subsequent *in vitro* studies, since the proliferation of this type of cell is one of the first phenomena seen in lupus nephritis pathogenesis.

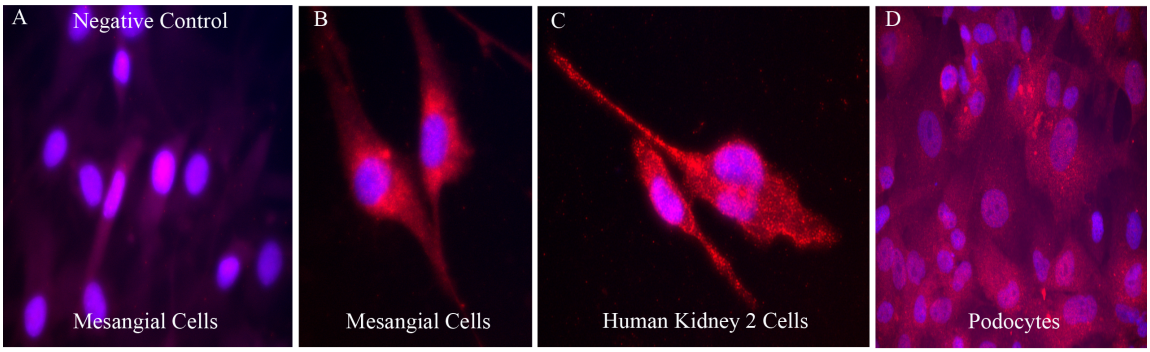


Figure 7.4 – Expression of HER2 in different cell types. A. Negative control without the primary antibody performed in mesangial cells. **B.** Human mesangial cells. **C.** Human kidney 2 cells. **D.** Human podocytes.

We found that mesangial cells exposed to IFNα indeed had significantly higher expression of HER2 than did control cells (P=0.02) (Figure 7.5D).

IRF1 is a key transcription factor induced by IFNα. We therefore decided to examine its effect on HER2 expression. IRF1 transfection was confirmed by immunofluorescence and qRT-PCR, as shown in Figure 7.5A. HER2 expression was significantly increased in IRF1–transfected cells (P=0.0009) (Figure 7.5B). Furthermore, decreased levels of miR-26a and miR-30b were seen in IRF1–transfected cells (Figure 7.5C). These data suggested that lupus-associated factors such as IFNα and IRF1 contribute to HER2 overexpression and to secondarily decreased miR-26a and miR-30b levels seen in lupus nephritis.

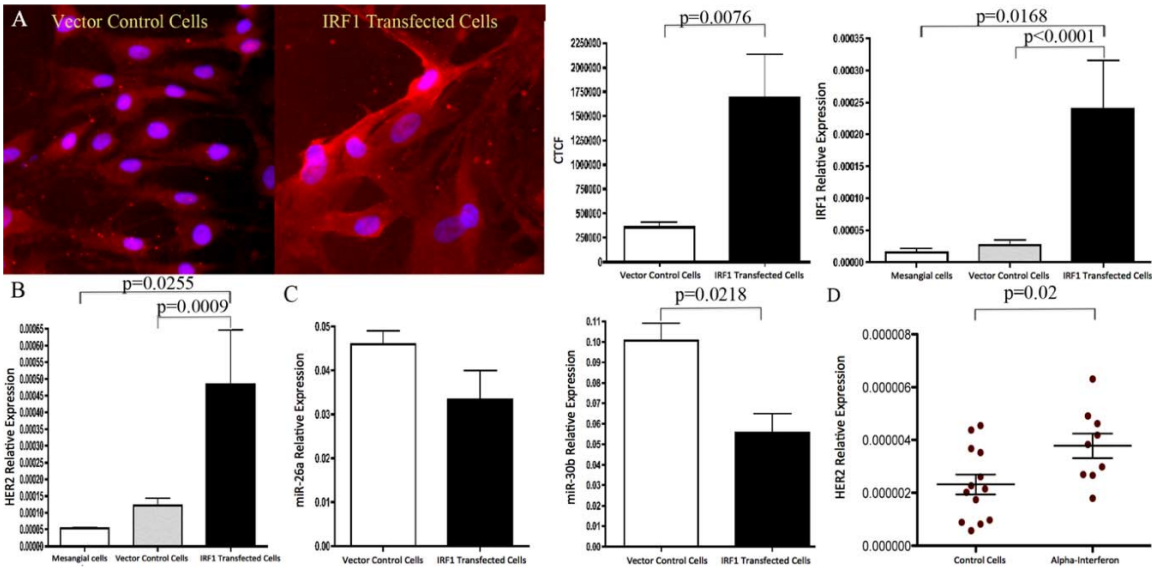


Figure 7.5 –IFNα and IRF1 increase the expression of HER2 in human mesangial cells. A. IRF1 expression in cells transfected with IRF1 and with a vector-control measured by immunofluorescence and qRT-PCR demonstrates successful overexpression. **B.** Expression of HER2 in mesangial cells transfected with IRF1 or the control vector was studied by qRT-PCR. **C.** MiR-26a and miR-30b levels were measured in cells transfected with IRF1 and vector-control by qRT-PCR. **D.** Expression of HER2 in mesangial cells, with and without previous IFNα exposure, was measured by qRT-PCR. Error bars indicate the standard error of the mean. The experiments were performed at least three times.

7.4.5 HER2 is increased in the urine of patients with lupus nephritis and is associated with disease activity

The dramatic overexpression of HER2 in lupus nephritis renal tissue suggested that it could be a useful biomarker. We, therefore, examined the urine from patients and controls. HER2 was found to be significantly increased in the urine of adult patients with active lupus nephritis when compared to sex and age-matched healthy controls (p=0.0002; Figure 7.6A). Moreover, when analyzing HER2 levels longitudinally, it was found that they were significantly increased during lupus nephritis flares (p=0.0270; Figure 7.6B). HER2 was also significantly increased in the urine of patients with class III and class IV lupus nephritis when compared with class V (p=0.0374; p=0.0004, respectively; Figure 7.6C). Finally, HER2 levels correlated with the urine protein: creatinine ratio (p=0.0227) and with the levels of other lupus nephritis described biomarkers, namely MCP1 (p=0.0304) and VCAM1 (p<0.0001) (Figure 7.6D).

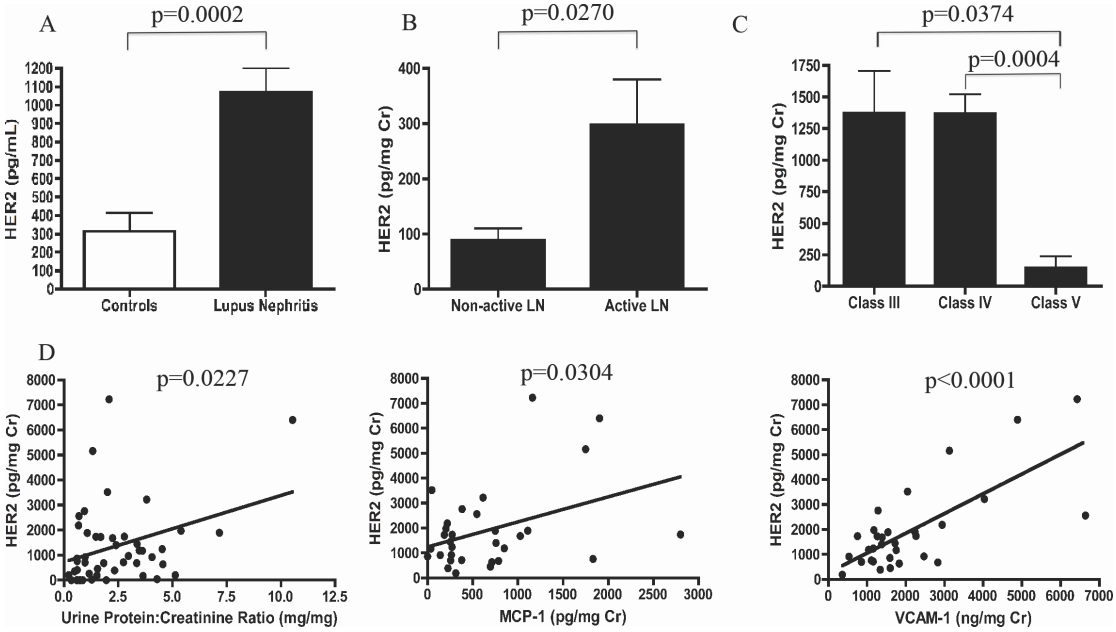


Figure 7.6 – HER2 is increased in the urine of patients with active lupus nephritis and it is associated with disease activity. **A.** HER2 levels (pg/mL) were measured by ELISA in the urine of adult patients with active lupus nephritis (N=47) and in sex and age-matched healthy controls (N=26). **B.** HER2 levels (pg/mg Cr) were analyzed in 14 patients from two different time-points: non-active lupus nephritis and a lupus nephritis flare. **C.** Urinary HER2 levels (pg/mg Cr) analyzed according to different histologic classes of lupus nephritis (N=19). **D.** HER2 levels (pg/mg Cr) were expressed according to the urine protein: creatinine ratio (mg/mg) (N=46), MCP1 (pg/mg Cr) (N=31) and VCAM1 levels (ng/mg Cr) (N=31). Error bars indicate the standard error of the mean.

7.5 DISCUSSION

Trastuzumab targets the HER2 extra-cellular domain and blocks its downstream pathways by inhibiting the homo/hetero dimerization of HER2 and by promoting the internalization and cleavage of HER2 molecules. Currently, trastuzumab is used for the treatment of patients with $HER2^{+}$ breast and gastric cancer. In breast cancer cells, it was previously found that trastuzumab caused a G1 arrest by up-regulating miR-26a and miR-30b (619). Since we identified a significant decrease in these two miRNAs in the kidneys of patients with lupus nephritis, we hypothesized that HER2 might also have a role in lupus nephritis pathogenesis. The immunohistochemistry studies showed indeed a dramatic increase of HER2 expression in the kidneys of NZM2410 mice and in lupus nephritis patients. Furthermore, mesangial cells exposed to trastuzumab had a down-

regulation of genes associated with mitosis and cell proliferation. Thus, HER2 may represent a new therapeutic target.

The Epidermal growth factor receptor (EGFR) family of transmembrane receptor tyrosine kinases is comprised of four members: EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4), where the HER and ErbB nomenclatures generally refer to human and mouse EGFR-family receptors, respectively. There are at least 11 ErbB ligands, including epidermal growth factor (EGF) itself. Depending on the ligand-receptor combination, signaling can involve MAP/ERK, PI3K/Akt, and JAK/STAT pathways – leading to cell survival, growth, and proliferation (664,665).

Although no data are available in human lupus nephritis, several murine models of other types of kidney disease indicate that the role of EGFR-family receptors in the kidney is complex. Early work suggested that exogenous EGF could promote renal repair in renal ischemia-reperfusion injury (666), mercury-induced renal damage (667), and unilateral ureteral obstruction (668). More recently, Chen and collaborators found that either erlotinib (an EGFR kinase inhibitor) or the genetic deficiency of EGFR in the renal proximal tubule cells delayed renal recovery after ischemia-reperfusion injury, suggesting that EGFRs are required for tissue repair in the kidney (669). EGFR may also have an important regulatory role as the EGFR ligand amphiregulin enhances regulatory T cell functions (670). These studies suggest that EGFR signaling is protective during kidney damage.

In contrast, other work suggests that EGFR-family signaling exacerbates kidney disease. EGFR inhibition with erlotinib limits angiotensin II – induced renal fibrosis (671); EGFR blockade reduces unilateral ureteral obstruction – induced renal fibrosis (672); and transgenic mice expressing a dominant-negative EGFR have reduced pathology in two different murine models of renal injury, subtotal nephrectomy and renal ischemic injury (673).

In a crescentic glomerulonephritis mouse model, the activation of EGFR in podocytes resulted in the development or progression of the disease, while the pharmacological blockage or genetic deletion of one of its ligands improved the course of the disease and prevented the infiltration of inflammatory cells (674). Moreover,

activation of EGFR in cultured podocytes led to proliferation, dedifferentiation and migration, processes that are thought to occur in crescent formation *in vivo* (674).

Current evidence suggests, therefore, that EGFR-family signaling mediates kidney disease. The disparate effects of EGFR-family signaling in murine kidney disease may have many technical causes, although some have suggested that EGFR-family signaling is protective in acute kidney injury and becomes maladaptive in chronic renal disease (675,676).

Since lupus nephritis often involves a chronic proliferative component, we hypothesized that pharmacologic blockade of EGFR-family receptors would limit maladaptive proliferation and renal disease in lupus nephritis.

Interestingly, we did not find HER2 overexpression in other types of glomerulonephritides also characterized by mesangioproliferation, such as IgA nephropathy, post-streptococcal glomerulonephritis and granulomatosis with polyangiitis. Accordingly, we hypothesized that lupus-associated factors drive HER2 overexpression. The effects of IFNα and IRF1 in human mesangial cells were evaluated, since the role of these two factors in SLE etiopathogenesis has been extensively studied (549,677,678). It is now well known that in SLE there is an IFN-inducible gene expression signature (279,677,679). Lupus prone mice also demonstrate this IFN signature, even before the onset of autoimmunity (680,681). Moreover, multiple groups have shown that knocking out type I IFN receptor (IFNAR) in lupus-prone strains largely stifles disease progression (682,683). In addition, a single iv dose of an adenoviral vector that expresses murine IFNα can drastically accelerate the onset of autoimmunity in lupus prone mice and the progress to nephritis (289,292,684).

As previously described in Chapter 3, SLE susceptibility is associated with functional polymorphisms in genes in the type I IFN pathway, including IRF5 (285,286,685–687) and IRF7 (287,688,689). Moreover, IRF1, an IFN-induced weak transcription factor, also seems to be associated with SLE pathogenesis. Previous studies performed in our laboratory documented an overall increase on histone 4 acetylation and, consequently, on global gene transcription in monocytes from patients with systemic lupus erythematosus. Interestingly, 63% of the genes with increased H4 acetylation had the potential for IRF1 regulation (549). The ChIP-Seq data available on

UCSC Genome Bioinformatics platform are consistent with the binding of IRF1 to the HER2 promoter region in K562 cells, which further supports our hypothesis that IRF1 controls HER2 expression.

We showed that both IFNα and IRF1 increased HER2 expression in human mesangial cells and that IRF1 was also associated with a significant decrease of miR-30b.

Notably, urinary HER2 was significantly increased in lupus nephritis patients and it was associated with proliferative disease, as expected from its known role in cell growth. Urinary HER2 levels were also significantly correlated with urine protein to creatinine ratio, MCP1 and VCAM1 levels, well established lupus nephritis biomarkers (690–692).

In the kidney, MCP1 is secreted in response to pro-inflammatory stimuli including TNF and it recruits inflammatory cells to the glomeruli (690). VCAM1 is a cell adhesion molecule involved in the acute phase of inflammation when leucocytes are being recruited to the kidneys. Urinary VCAM1 levels are strongly associated with the renal pathology activity index and class IV and are negatively correlated with the chronicity index. VCAM1 is, therefore, a reliable indicator of the activity: chronicity ratio (691,693).

The correlation between HER2 and MCP1 and HER2 and VCAM1 shows that the etiopathogenesis of lupus nephritis is complex and that the different mechanisms that contribute to kidney damage, namely cell proliferation, chemotaxis and invasion of inflammatory cells, are all interconnected. Moreover, the disruption of the normal kidney architecture contributes to the occurrence of kidney damage and proteinuria, being HER2 levels also associated with the proteinuria: creatinuria ratio.

HER2 levels likely capture a distinct pathologic mechanism in lupus nephritis and while the association between biomarkers is statistically robust, there are clear differences between the inflammatory mediator detection and HER2 detection, which will require additional investigation.

This study identified potential biomarkers and a novel therapeutic target using multiple confirmatory strategies. There are, however, some limitations. While our initial miRNA analysis and pathologic studies relied on pediatric cases of lupus nephritis, the urine analyses were performed in adults where achieving an adequate sample size was possible. This precluded direct analysis of the association of HER2 and miRNA expression from the same patient.

In conclusion, with this study we identified the down-regulation of miR-26a and miR-30b in the kidneys and urine of lupus nephritis patients and we showed that these miRNAs are associated with the control of mesangial cell proliferation. In addition, we analyzed the expression of HER2, a known regulator of miR-26a and miR-30b levels in breast cancer cells. We reported the existence of a significant overexpression of HER2 in the kidneys of lupus nephritis patients and in a lupus nephritis mouse model, but not in other proliferative glomerulonephritides. *In vitro*, we identified that exposure to IFNα and transfection with IRF1 caused a significant increase in HER2. These factors can, therefore, contribute for the HER2 overexpression seen in lupus nephritis. Finally, we reported that HER2 was not only increased in the urine of patients with active lupus nephritis, but also correlated with disease activity and the levels of other lupus nephritis biomarkers. These findings raise the possibility of urinary miR-26a, miR-30b and HER2 being useful biomarkers of lupus nephritis activity and establish strong foundations for the use of anti-HER2 drugs for cell proliferation control in this disease.

7.6 CONCLUSIONS

Our data support a model where type I IFNs, via the transcription factor IRF1, induce the expression of HER2. Either directly or indirectly, this pathway downregulates miR-26a and miR-30b, thereby driving cell proliferation through de-repression of genes involved in the cell cycle. For decades, proliferation has been recognized as the hallmark of lupus nephritis with a poor prognosis and the identification of this pathway established strong foundations for the use of urinary miR-26a, miR-30b and HER2 as biomarkers of lupus nephritis activity and opens the door for novel therapeutic interventions.

Please refer to **Appendix A.3** for the paper ***The role of MicroRNAs and Human Epidermal Growth Receptor 2 in Proliferative Lupus Nephritis***, which was published in the journal *Arthritis and Rheumatology*.

CHAPTER 8

A New Insight Into the Role of Hemophagocytes

8.1 INTRODUCTION

8.1.1 Overview

Hemophagocytic syndromes are characterized by overwhelming inflammation due to massive secretion of proinflammatory cytokines and uncontrolled activation of macrophages and T lymphocytes (694–696). The hallmark of these syndromes is the appearance in the bone marrow of hemophagocytes: activated macrophages that have engulfed other hematopoietic cells.

Hemophagocytic syndromes can be primary, also known as familial hemophagocytic lymphohistiocytosis, a group of rare genetic diseases caused by impaired cellular cytotoxicity, or secondary to multiple inflammatory disorders, including infections, malignancies, and rheumatic diseases. The term macrophage activation syndrome is often used when referring to the latter.

Hemophagocytic syndromes are vastly under-recognized, particularly in the intensive care unit, where they may be labeled as multiple organ dysfunction or systemic inflammatory response syndrome (697). The identification of this condition is particularly important, since the mortality rates are high and the treatment strategy often includes immunosuppressive agents, a radically different approach from other causes of multi-organ dysfunction.

8.1.2 Clinical manifestations

A high level of clinical suspicion is necessary for diagnosis. Typically, patients become acutely ill with the sudden onset of non-remitting high fever, cytopenias, liver function abnormalities, neurologic symptoms and multiorgan failure (698–700).

Notably, in patients with systemic juvenile idiopathic arthritis (sJIA) the high spiking, intermittent, fever pattern, typical of active disease, shifts to a continuous non-remitting pattern at the onset of the macrophage activation syndrome (698).

The most common clinical manifestations are hepatosplenomegaly and generalized lymphadenopathy (698–703).

Skin manifestations can be varied. These include rashes, petechiae and purpura (699,703). Patients may also have easy bruising and mucosal bleeding (698–700,703). The hemorrhagic diathesis may be caused by altered coagulation due to liver failure,

thrombocytopenia from bone marrow failure or platelet function defects associated with an underlying genetic defect in platelet granule processing (704).

Central nervous system dysfunction occurs frequently and may cause headache, lethargy, irritability, disorientation, ataxia, focal neurologic deficits, sensory and motor peripheral neuropathy, seizures or coma (698–700). Sometimes these findings dominate the clinical picture or develop prior to the appearance of other signs or symptoms (705–708).

Recently, it was reported that lung involvement was also common in patients with secondary forms of hemophagocytic syndromes and it was a poor prognosis factor (709). Dyspnea and cough were the most frequent symptoms (709).

Other possible symptoms include serositis, myocarditis, cardiomegaly, arrhythmia and heart failure (698,700,702).

Finally, renal involvement can also occur in patients with hemophagocytic syndromes (698,700). These patients frequently progress to renal failure and multi-organ failure (710,711).

8.1.3 Laboratory abnormalities

Frequent laboratory abnormalities seen in patients with hemophagocytic syndromes include pancytopenia and elevated ferritin (699,700,703,712).

In a large cohort study, ferritin levels greater than 500, 5,000, and 10,000 ng/mL were seen in 93%, 42%, and 25%, respectively (713). The median ferritin level was 2,950 ng/mL (713). In another cohort, a ferritin level higher than 500 ng/mL was 100% sensitive for a hemophagocytic syndrome, but less specific, while a ferritin level higher than 10,000 ng/mL was 90% sensitive and 96% specific, with very minimal overlap with sepsis, infections and liver failure (714). Notably, hyperferritinemia is not only an important laboratory hallmark of hemophagocytic syndromes, but also a useful indicator of disease activity, therapy response and prognosis (699,703).

Liver enzyme levels greater than three times the upper limit of normal have been seen in 50% to 90% of patients with hemophagocytic syndromes (715,716). Higher bilirubin and gamma glutamyl transferase levels have also been reported (699,703,712), reflecting the biliary tract infiltration by lymphocytes and macrophages (717). Lactate

dehydrogenase is elevated in 85% (716). Hypertriglyceridemia occurs in 68% of patients and it may be due to severe liver involvement (718).

In patients with hemophagocytic syndromes there is usually an abnormal coagulation profile, with prolonged prothrombin time and prolonged partial thromboplastin time, hypofibrinogenemia and the presence of fibrin degradation products (698,699,703,712).

Low sodium and albumin levels are also observed consistently (699,702,703). A decrease in the erythrocyte sedimentation rate (ESR) is common in hemophagocytic syndromes and should raise the suspicion of this diagnosis, particularly in patients with sJIA who usually have an elevated ESR (699,703,712). A paradoxical decrease on the ESR in a patient who clinically has more signs of inflammation should, therefore, prompt the necessary laboratory tests to identify a macrophage activation syndrome. It has been suggested that a ratio ferritin to ESR higher than 80 has a high sensitivity and specificity to differentiate between new-onset sJIA and macrophage activation syndrome (719) .

New markers of hemophagocytic syndromes have been recently described. The soluble IL2 receptor α (sIL2Rα / sCD 25) is highly increased in the acute phase of macrophage activation syndrome compared to untreated new-onset sJIA (720).

CD163, a haptoglobin scavenger receptor, which is a specific marker for macrophages, is highly increased in serum, bone marrow and in the spleen of patients with hemophagocytic syndromes (720,721). Furthermore, sCD25 and soluble CD163 were found to correlate with disease activity, reflecting the degree of activation of T lymphocytes and macrophages, respectively (720).

FSTL1 is a protein produced by cells of mesenchymal origin that is thought to be a mediator of the innate immunity pathways that cause arthritis in sJIA (722–724). FSTL1 was found to be elevated in active sJIA and to be even more increased during macrophage activation syndrome (719). The levels of FSTL1 correlated with the levels of sCD25 and ferritin and normalized after treatment (719).

Neopterin is an acute phase reactant produced by activated macrophages as a byproduct of nitric oxide synthesis. Neopterin is increased in patients with hemophagocytic syndromes and its levels also correlated with ferritin levels (725).

CD107a or LAMP1 is a membrane protein expressed on the surface of cytotoxic cells following degranulation. Some patients with familial hemophagocytic

lymphohistiocytosis have genetic defects that impair degranulation which cause defective CD107a mobilization to the cell surface. Evaluation of CD107a mobilization upon *ex vivo* stimulation is, therefore, a rapid functional assay to study defects in degranulation (726,727).

8.1.4 Pathology

A bone marrow examination is often used in the diagnostic algorithm of a critically ill patient with cytopenias. The bone marrow specimens should be cultured and examined for infectious organisms and evidence of lymphoid malignancy. In hemophagocytic syndromes the macrophages do not have the atypical features associated with malignant histiocytes and they are clearly different from the CD1a-staining Langerhans cells identified in patients with Langerhans cell histiocytosis. In addition to CD68 immunostaining, it is recommended to stain the bone marrow for the hemoglobin-haptoglobin scavenger receptor CD163 to better identify the hemophagocytes (712).

The presence of hemophagocytosis in the bone marrow aspirate or biopsy might be helpful, but it is not sufficient or necessary for the diagnosis of a hemophagocytic syndrome.

Notably, hemophagocytosis is not always demonstrable in the initial stages of the disease (712). A study of 21 pediatric marrow specimens showed that the numbers of hemophagocytes were often initially low or even absent in patients with a hemophagocytic syndrome and that sensitivity of these marrow findings was 58% (728). Hemophagocytic activity is usually a rather late sign, occurring in an advanced stage of a hemophagocytic syndrome (729). Diagnosis and treatment should never be delayed due to the lack of bone marrow hemophagocytosis.

Furthermore, patients with sJIA may have occult hemophagocytosis without any clinical evidence of macrophage activation syndrome (712). Recently, a study compared the quantity of hemophagocytes in 58 adult patients with and without secondary hemophagocytic syndromes, many of whom initially presented with similar signs and symptoms (730). It was found that marrow histologic findings alone did not reliably predict the probability of a hemophagocytic syndrome and an isolated finding of hemophagocytosis, even when present in a high amount, lacked specificity (730).

In conclusion, pathology may be considered when the diagnosis is doubtful, but it is not mandatory.

8.1.5 Differential diagnosis

A hemophagocytic syndrome may be triggered by a multiplicity of stimuli, including a rheumatic disease, a malignancy, an infection or an immunodeficiency. It can also occur due to genetic defects in cytotoxicity, as is the case with familial hemophagocytic lymphohistiocytosis. Patients with one of these rare diseases start with symptoms during infancy or early childhood. The median survival is less than 2 months after diagnosis if untreated (731). The onset of primary hemophagocytic syndromes in adolescents and adults has more recently been reported and is associated with a milder phenotype (732).

Regarding rheumatic diseases, macrophage activation syndrome is particularly common in patients with sJIA, occurring in at least 7% to 13% of patients (698,701,712). Some studies suggest that the syndrome may occur subclinically or in a mild form in another 30% to 40% of patients (712,720). The reported prevalence in patients with SLE is lower, ranging from 0.9% to 4.6% (733). Macrophage activation syndrome can also occur in patients with Sjögren disease (734), Sarcoidosis (734), Kawasaki Disease (734,735), Periodic Fever Syndromes (701,736), Dermatomiositis (734,737), Systemic Sclerosis (734), Polyarteritis Nodosa (734), Rheumatoid Arthritis (734), Enthesitis Related Arthritis (701,734), and adult-onset Still disease (734).

In a large retrospective study, the median time interval between the onset of sJIA and the occurrence of macrophage activation syndrome was 4 months (700). It is clear that, even though macrophage activation syndrome can occur in patients with longstanding rheumatic diseases or be the first manifestation of a rheumatic disease (698,700,701,735), it tends to occur in the earlier stages of the disease (700).

Macrophage activation syndrome may occur spontaneously or may be triggered by an infection, a change in the medication regimen or by the toxic effect of a drug (700,701).

In addition to rheumatic diseases, malignancies can also trigger a hemophagocytic syndrome, frequently also in association with an infectious trigger (738). The likelihood of an underlying malignant disease increases with age (738). The most frequently

associated malignancies are T lymphocyte and Natural Killer Cell leukemias and lymphomas, diffuse large B lymphocyte lymphoma and Hodgkin lymphoma (739–741). In children, T cell malignancies predominate, namely peripheral T cell lymphomas, particularly subcutaneous panniculitis-like T cell lymphoma, primary cutaneous YδT cell lymphoma, anaplastic large cell lymphoma and, less commonly, lymphoblastic lymphomas (738,742). The hemophagocytic syndrome may occur at the onset of the identification of the malignancy (741) and it can also happen in the setting of iatrogenic immunosuppression from chemotherapy (738).

Viral infections, particularly Epstein-Barr virus, may trigger primary as well as secondary forms of hemophagocytic syndromes (698,743,744). Other known viral triggers include cytomegalovirus (744), parvovirus B19 (698,745), herpes simplex virus (746,747), varicella-zoster (698,748), human herpes virus 8 (749,750), coxsackie virus (698), influenza virus (751,752), parainfluenza virus (753), parechovirus (754) and human immunodeficiency virus (HIV) (755) alone or in combination.

Interestingly, it was shown that among fatal cases of influenza A (H1N1) infection there was a high prevalence of mutations associated with familial hemophagocytic lymphohistiocytosis, including mutations in *PRF1* and *LYST* (756). It was, therefore, suggested that these mutations are risk factors for mortality among individuals with influenza A (H1N1) infection (756). This implies that the individual's genetically determined immune response plays an important role in the development of an infectious triggered hemophagocytic syndrome.

Although less common, hemophagocytic syndromes can also occur in the setting of bacterial infections, including infections caused by *Staphylococcus aureus (744)*, *Escherichia coli* (744), *Salmonella enteritidis* (698), *Mycoplasma pneumoniae* (757), *Ehrlichia chaffeensis (758)*, *Brucella mellitensis* (744) and *Mycobacterium tuberculosis* (744).

Parasites, including *Leshmania amastigotes* (744) and *Plasmodium falciparum* (759) and *vivax* (760), can also trigger a hemophagocytic syndrome, as well as fungi, including *Histoplasma capsulatum* (744,761) and *Coccidiodes (762)*.

Patients with X-linked lymphoproliferative syndromes are particularly vulnerable to Epstein-Barr infections and to develop a hemophagoytic syndrome when exposed to this virus (763).

In the course of Langerhans cell histiocytosis, Chédiak-Higashi Syndrome (764), Griscelli type 2 syndrome (765) and Hermanski-Pudlak type 2 syndrome (766) a hemophagocytic syndrome can occur.

Acquired immunodeficiencies have also been associated with the occurrence of hemophagocytic syndromes, including HIV/AIDS, hematopoietic cell transplantation (767) and kidney and liver transplantation (768–770). The hemophagocytic syndrome may occur in the setting of a concurrent infection or a lymphoproliferative disease (770).

Patients with lysinuric protein intolerance (771), severe combined immunodeficiency (772), Omenn syndrome (772,773) and chronic granulomatous disease (772) may present in a similar way to patients with familial hemophagocytic lymphohistiocytosis. Usually infections are the triggers for the hemophagocytic syndrome in patients with primary immunodeficiencies (772). Interestingly, hemophagocytic syndromes have not been described in other congenital defects in phagocytes and only rarely in other defects in innate immunity (772).

There are several conditions that may resemble a hemophagocytic syndrome. The autoimmune lymphoproliferative syndrome (ALPS) is one of them. ALPS is caused by genetic defects in the machinery of FAS-mediated apoptosis, which leads to expansion of some autoreactive lymphocyte populations. Patients may, therefore, present with hepatosplenomegaly, rash, and autoimmune cytopenias. An autoimmune hepatitis or a Guillain Barré syndrome may also occur and may contribute to the difficult differential diagnosis. Patients with ALPS, however, typically do not have multiorgan failure, extremely high ferritin levels or severe liver failure.

Thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, or drug-induced thrombotic microangiopathy may also present with similar manifestations. These conditions are characterized by endothelial damage, microvascular thrombosis, and anemia. Fever, neurologic findings, or renal failure may occur. Patients with these conditions generally do not have rising ferritin or liver function abnormalities and the anemia is microangiopathic.

Primary liver disease, like hemophagocytic syndromes, can present with hepatomegaly and elevated liver function tests. Both can cause a coagulopathy with prolonged prothrombin time and prolonged partial thromboplastin time, low fibrinogen, and elevated D-dimer and both can cause encephalopathy. Patients with

hemophagocytic syndromes typically have more extensive organ involvement, cytopenias, extremely high ferritin, and neurologic findings.

In conclusion, the diagnosis of a hemophagocytic syndrome is indeed challenging. The physician must differentiate it from other conditions that may have similar presentation, must distinguish between primary and secondary forms, and must perform a systematic search of a possible trigger. Only with this approach the prompt and effective treatment can be implemented, which will have a profound impact on survival.

8.1.6 Diagnostic criteria

The criteria proposed by the Histiocyte Society in 2004, followed by specific genetic testing, remain the gold standard for the diagnosis of primary hemophagocytic syndromes (774). These criteria include: fever, splenomegaly, bicytopenia, hypertriglyceridemia and/or hypofibrinogenemia, hyperferritinemia, high sIL2Rα/sCD25, low/absent natural killer cell activity and hemophagocytosis in the bone marrow, spleen or lymph nodes. Altogether five of these eight criteria have to be fulfilled, unless familial history or molecular diagnosis is consistent with familial hemophagocytic lymphohistiocytosis (774). These criteria are not tailored, however, for the diagnosis of other hemophagocytic syndromes, since they do not perform well in distinguishing macrophage activation syndrome from a flare of rheumatic diseases or from sepsis.

In 2009, the Pediatric Rheumatology European Society published the results of a multinational, multicentric study of patients with juvenile onset SLE who developed macrophage activation syndrome (775). Based on the conclusions of this study, diagnostic criteria for macrophage activation syndrome in SLE were suggested:

- Clinical criteria
  - Fever (>38°C);
  - Hepatomegaly (≥ 3 cm below the costal arch);
  - Splenomegaly (≥ 3 cm below the costal arch);
  - Hemorrhagic manifestations and
  - Central nervous system dysfunction
    (irritability, disorientation, lethargy, headache, seizure or coma)
- Laboratory criteria
  - Cytopenia affecting two or more cell lineages

(white blood cell count ≤4.0 x 10^9/L; hemoglobin ≤9.0 g/dL or platelet count ≤150 x 10^9/L);

- Increased aspartate aminotransferase (>40 U/L);
- Increased lactate dehydrogenase (>567 U/L);
- Hypofibrinogenemia (fibrinogen ≤1.5g/L) ;
- Hypertriglyceridemia (triglycerides >178 mg/dL) and
- Hyperferritinemia (ferritin >500 μg/L).

- Histopatologic criteria
  - Evidence of macrophage hemophagocytosis in the bone marrow aspirate.

The diagnosis of macrophage activation syndrome requires the simultaneous presence of at least one clinical criterion and at least two laboratory criteria (775). Bone marrow aspiration is only considered in these recommendations in doubtful cases (775).

It is particularly difficult to distinguish between macrophage activation syndrome and a flare of sJIA, since they have a similar clinical presentation. The criteria from the International League of Associations for Rheumatology for the diagnosis of sJIA include fever, hepatosplenomegaly and lymphadenopathy, all of which are also hallmarks of macrophage activation syndrome (776). Patients with this disease also have elevated ferritin levels (777), D-dimers (778) and prothrombin time (779). There is, therefore, a large degree of overlap between both entities. Moreover, it was found that 53% of sJIA patients, who did not have clinical evidence of macrophage activation syndrome, had hemophagocytosis on the bone marrow aspirate (712). It was thus hypothesized that sJIA and macrophage activation syndrome could be a single entity within a spectrum of severity, with fulminant macrophage activation syndrome being the most severe form (712).

Recently, new clinical and laboratory criteria were established for the diagnosis of macrophage activation syndrome in the context of sJIA (780). According to these criteria (780), a febrile patient with known or suspected sJIA should be classified as having macrophage activation syndrome if the following conditions are met:

- Ferritin >684 ng/mL
- and any two of the following:
  - Platelet count ≤181 x 109/L;
  - Aspartate aminotransferase>48 U/L;

- Triglycerides >156 mg/dL or
- Fibrinogen ≤360 mg/dL.

These criteria were established using a consensus process and a statistical approach comparing groups of patients with or without macrophage activation syndrome in the context of rheumatological diseases. Interestingly, the criteria do not include clinical manifestations, with the exception of fever. This reflects the notion that clinical symptoms are often delayed and similar to those observed in other conditions. The diagnosis of macrophage activation syndrome should be, therefore, raised by detection of subtle laboratory alterations (780). In fact some of the cut-off values of the variables included in the criteria are within the normal range. This is explained because sJIA patients usually have elevated levels of inflammatory markers, like fibrinogen (>500-600mg/dL). A paradoxically normal fibrinogen level in the setting of prominent inflammation should, therefore, raise the suspicion of macrophage activation syndrome. Notably, the demonstration of hemophagocytosis is not mandatory once again for the diagnosis.

8.1.7 Clinical approach

Patients should be assessed as quickly as possible and start treatment promptly. Patients and families should be questioned about recent infections, malignancy, immunodeficiencies, rheumatic diseases, pharmacologic immunosuppression and family history of similar symptoms. Every patient should be evaluated for hepatosplenomegaly, lymphadenopathy, rashes, bleeding and neurologic abnormalities.

An abdominal ultrasound should be performed (781). All patients should have a basic metabolic panel, coagulation studies, liver function tests, lactate dehydrogenase, albumin, lipid profile and ferritin (781).

If no trigger is identified, a work up for infectious diseases should be performed, including chest x-ray, blood and urine cultures, blood smear, viral titers and serology for Epstein-Barr virus, cytomegalovirus, herpes simplex virus, varicella zoster virus, parvovirus, influenza, adenovirus, hepatitis B, hepatitis C virus and HIV (781).

To exclude a malignancy peripheral flow cytometry, imaging studies and bone marrow biopsy are appropriate (781).

Flow cytometry screening for perforin deficiency is recommended (782). If the screening results suggest it, it should be performed a genetic testing for the known familial hemophagocytic lymphohistiocytosis associated mutations, including *PRF1, UNC13D, STX11, STXBP2* and *RAB27A* (781). In anticipation of a failure of treatment response, HLA typing and echocardiogram are recommended (781).

8.1.8 Treatment

Since hemophagocytic syndromes are rapidly progressive and often life-threatening conditions, a prompt initiation of treatment is essential for the survival of affected patients. A complete mechanistic understanding of the different types of hemophagocytic syndromes is needed in order to better select effective therapeutic strategies. Until new data are available regarding the pathogenesis of these syndromes, treatment strategies are based on anecdotal experience and consensus protocols.

In patients who are clinically stable and have a recognized trigger for the hemophagocytic syndrome, it is recommended to initiate treatment directed to the triggering condition. Infection should be diagnosed rapidly and empiric antibiotic, antifungal, antiviral or antiparasitic therapy should be started according to the suspected organism. When Epstein-Barr infection infection is identified, rituximab 375 mg/m^2 weekly for one to four weeks is recommended.

The Histiocyte Society Protocol for the treatment of Hemophagocytic Lymphohistiocytosis (HLH-94) is the most well-known and used treatment strategy. This protocol was used in a large prospective pediatric study conducted by the Histiocyte Society in patients with less than 16 years with no history of malignancy or immunosuppression. It includes an eight-week induction phase with dexamethasone and etoposide (706). If neurological symptoms are present, patients also receive intrathecal methotrexate. This treatment strategy was effective in prolonging survival of patients with familial hemophagocytic lymphohistiocytosis, in some cases more than 5 years after onset (783). Ultimately, allogenic hematopoietic stem cell transplantation can provide the cure for this condition (784–786).

This protocol underwent a small revision (HLH-2004), in which cyclosporine A was included in the induction phase. Notably, the triple drug induction with etoposide, dexamethasone and cyclosporine A was shown to produce overt neurotoxicity, so

current recommendations favor an induction phase with etoposide and dexamethasone alone, followed by cyclosporine A maintenance (787).

In 2015, the first prospective clinical trial was performed in adults with hemophagocytic syndromes, triggered mainly by malignancy or infection (788). In this study etoposide and dexamethasone were used in the induction phase and if the patients did not achieve partial remission after two weeks, treatment with doxorubicin, etoposide and methylprednisolone was initiated (788). An overall response of 76% was achieved, which seems promising (788).

Patients with macrophage activation syndrome should be treated initially with steroids alone and typically do not require etoposide. When the response is not adequate, additional immunosuppressive agents tailored to the underlying rheumatic disease should be used rather than the HLH-94 or the HLH-2004 protocol (781).

Full remission on steroid monotherapy has been achieved in many episodes of macrophage activation syndrome (698,789), with remission rates of up to 71% (698,789,790).

Cyclosporine A suppresses T lymphocyte activation, reduces the macrophage production of inflammatory cytokines and inhibits the expression of cell surface costimulatory molecules in dendritic cells (791–794). It has been shown that cyclosporine A is effective in the treatment of primary hemophagocytic syndromes and of severe or corticosteroid-resistant macrophage activation syndromes (698,700,701,795–798). In some patients this drug causes resolution of fever and improvement of the laboratory abnormalities within 12 to 24 hours (796). These data led some authors to propose using cyclosporine A as the first-line treatment in macrophage activation syndrome (dose range 2-5 mg/Kg/day) (796,799).

The first cytokine inhibitors used to treat macrophage activation syndrome were the TNF blockers (800–803). The early enthusiasm has vanished, however, since several reports have linked the use of TNF blockers with the occurrence of macrophage activation syndrome (804–807).

IL1 and IL6 blockade seem more promising. Anakinra, an IL1 receptor antagonist, is highly effective in improving both systemic and arthritic features in patients with sJIA (808). Moreover, several patients with macrophage activation syndrome, complicating sJIA, have improved dramatically with anakinra (809–813). In contrast, there are reports

linking the use of anakinra to macrophage activation syndrome occurrence at dosages of 1-2mg/Kg/day (808,814). The exact cause and effect was difficult to establish and permanent discontinuation of anakinra was unnecessary for any of the patients. This may be, therefore, a case of confouding by indication. In fact, some of these patients improved on higher doses of anakinra. Considering the overall published experience with this drug, anakinra is, therefore, recommended for the treatment of refractory macrophage activation syndrome (695).

Another IL1 blocker, canakinumab, a monoclonal antibody that blocks IL1β, has been shown to reduce disease activity in patients with sJIA (815). Canakinumab has also been used for the treatment of macrophage activation system at higher doses (7.5-12.5mg/Kg) than usually utilized for the treatment of sJIA (4 mg/Kg), with rapid and complete resolution of symptoms and without side effects [1].

A recent study showed that treatment with canakinumab does not significantly change the risk of macrophage activation syndrome (816), since the latter occurs even in patients whose sJIA is well controlled with canakinumab (816). Infections remain the most common trigger in this group of patients (816).

Tocilizumab, a monoclonal antibody against IL6 receptor, has been shown to be highly effective in the treatment of sJIA (817,818). It is not clear if tocilizumab is as helpful for patients with macrophage activation syndrome. Furthermore, there have been several cases of macrophage activation syndrome attributed to IL6 blockade (819–821).

As previously stated, there is some concern that biological therapies can trigger hemophagocytic syndromes (710). Recently, it was reported the clinical characteristics and outcomes of 30 adult patients who developed a hemophagocytic syndrome after receiving biological therapies. It was found that biologic agent-induced infections were more likely to be the cause of the hemophagocytic syndrome than the drug itself (744).

Rituximab, a monoclonal antibody against CD20, is a B cell depleting agent, originally developed for B cell lymphoma treatment. Rituximab has been shown to be helpful for the treatment of multiple diseases, including SLE and refractory sJIA (822–

[1] A report in 2014, by Kostik and collaborators, was published on a Russian scientific journal, not available on Pubmed. This report described two patients who developed macrophage activation syndrome while being treated with canakinumab (4mg/Kg) for sJIA. Both patients were treated with higher doses of canakinumab (7.5 and 12.5mg/Kg) and had a fast recovery.

824). Rituximab has also been used to effectively treat Epstein-Barr associated hemophagocytic syndrome (825).

Etoposide was also able to induce rapid recovery in cases of steroid and cyclosporine A resistant macrophage activation syndrome (798,813). Nevertheless, with the availability of biologic agents, etoposide is nowadays used less frequently to treat macrophage activation syndrome, since combining corticosteroids, cyclosporine A and etoposide carries a not insignificant risk of death (826). Etoposide is, therefore, reserved for cases of familial hemophagocytic lymphohistiocytosis or secondary hemophagocytic syndromes triggered by malignancies or infections or in a severely ill patient refractory to treatment.

In conclusion, corticosteroids and cyclosporine A are the therapeutic agents more frequently used for the treatment of macrophage activation syndrome. Most clinicians start with intravenous methylprednisolone pulse therapy (30mg/Kg) for three consecutive days, followed by 2-3mg/Kg/day in 2-4 divided doses. If response to corticosteroids is not immediately evident, parenteral administration of cyclosporine A (2-5mg/Kg/day) is initiated (827). In patients refractory to this treatment, there is a growing body of evidence that biological therapies, particularly IL1 inhibitors, are useful (828).

Patients with gene mutations consistent with familial hemophagocytic histiocytosis, hematologic malignancies, relapsing symptoms, and/or central nervous system disease will require allogenic hematopoietic stem cell transplantation. It is recommended, therefore, that all patients and appropriate family members should undergo HLA typing and testing for familial hemophagocytic histiocytosis associated mutations in order to exclude that the donor does not have an undiagnosed condition. The prognosis is better if the patient is in remission at the time of the transplant (713,826). Alemtuzumab, a monoclonal antibody against CD52, a protein expressed on the surface of T and B lymphocytes, natural killer cells, monocytes, macrophages, dendritic cells and eosinophils, has shown promise in patients with refractory disease and has become standard peritransplant therapy (829,830). Reduced intensity conditioning before transplantation with alemtuzumab, fludarabine and melphalan is associated with lower toxicity risk and higher likelihood of survival (831,832).

Novel treatment modalities, such as gene therapy for correction of perforin defects, are currently being developed. Preliminary data on perforin gene transfer into hematopoietic stem cells of a mouse model of perforin-deficiency have shown encouraging results (833,834).

In two different mouse models of primary and secondary hemophagocytic syndromes, treatment with ruxolitinib, a Janus kinases (JAK) 1 and 2 inhibitor, lessened the clinical and laboratory manifestations, including weight loss, organomegaly, anemia, thrombocytopenia, hypercytokinemia and tissue inflammation. These data support the use of JAK inhibitors in future clinical trials (835).

Currently, an anti-human IFNγ monoclonal antibody is in phase I trial for patients with reactivated familial hemophagocytic lymphohistiocytosis. Patients with secondary forms were excluded. It is known that blocking IFNγ can increase susceptibility to infections. This could be deleterious in patients with recurrent hemophagocytic syndromes, since they are often triggered by infections.

In the past decade, based on the knowledge gained from basic science, anti-cytokine therapies have been used sporadically in different forms of secondary hemophagocytic syndromes and now we are, excitingly, entering an era where placebo-controlled trials of anti-cytokine therapy are being initiated for hemophagocytic syndromes (836). Hopefully all this effort will result in better survival for the patients.

8.1.9 Prognosis

Timely diagnosis and prompt treatment are essential to avoid progressive multiorgan failure and death related to hemophagocytic syndromes. Those with an inherited mutation in a gene associated with familial hemophagocytic lymphohistiocytosis had a survival of approximately two months after diagnosis, if not treated (731,783). Patients with a hemophagocytic syndrome treated with the 1994 protocol from the Histiocyte Society had a median survival of 54% at 6.2 years (713,826). Those younger than 6 months of age and those with neurological involvement had a lower survival (713,826). No patient with familial disease survived without hematopoietic cell transplant (713,826). Of the 124 patients who underwent allogeneic hematopoietic cell transplant the five-year survival was 66% (713,826). Survival was better in the group of patients who were in remission at the time of transplant (713,826). Long-term

sequelae of disease and/or treatment included late neurologic effects that ranged from severe mental retardation to learning disabilities.

The survival is much better if the hemophagocytic syndrome occurs in a patient with an underlying rheumatic disease. Recent studies found a mortality rate of 8% in patients with sJIA who develop macrophage activation syndrome (700).

Very high ferritin levels and failure of the ferritin to fall dramatically with treatment are poor prognostic signs across all types of hemophagocytic syndrome (837).

Relapses can occur, particularly in patients with familial hemophagocytic lymphohistiocytosis. Relapse is more likely within a year of the initial acute illness. The risk of relapse should be minimized by reducing exposure to triggering conditions. Since recurrences following vaccination have been reported, it is recommended to avoid vaccination for the first six months after treatment and then administer vaccinations one at a time (838).

Improvements in survival are expected to come from increased disease identification, earlier diagnosis and the development of disease specific immunotherapies.

8.1.10 New insight into the pathogenesis of hemophagocytic syndromes

In 1998, it was found that patients with familial hemophagocytic lymphohistiocytosis had defective natural killer cell function (839). Soon afterwards, it was described for the first time the association with perforin gene mutations (840). It is estimated that 15%-40% of patients with familial hemophagocytic lymphohistiocytosis have mutations in the gene encoding perforin (840).

Perforin is localized in the granules of natural killer cells and cytotoxic T lymphocytes and is secreted upon conjugation between effector and target cells. In the presence of calcium, it is able to insert into the membrane of the target cells, where it polymerizes to form a cell death-inducing pore. The effector molecule, granzyme B, a serine protease also found in cytotoxic cells, is delivered into target cells via the porelike structures produced by perforin and induces apoptosis.

Data from animal models indicate that perforin is important for the defense against cancer and intracellular pathogens (841,842). It has also been suggested that

perforin controls lymphocyte proliferation (840,843). Recently, it has been shown that defects in perforin-mediated killing of target cells prolongs the synapse time between the killer and its target cell by 5-fold and this results in a proinflammatory cytokine storm (844). A perforin deficiency may therefore contribute to persistent lymphocyte activation and, consequently, to the production of proinflammatory cytokines, including IFNγ, that activate macrophages. These cells may then produce high levels of pro-inflammatory cytokines.

Mutations in the gene *UNC13D,* which encodes the protein MUNC13-4, have been implicated in 10%-30% of patients with familial hemophagocytic lymphohistiocytosis (845). This protein participates in the docking and fusion of the cytotoxic granules with the cytoplasm membrane.

Mutations in *STX11* (Syntaxin 11) and *STXBP2* (Syntaxin Binding Protein 2), two genes encoding proteins important for the trafficking of cytotoxic granules, have also been linked to the development of primary hemophagocytic syndromes (846–848). Other genes essential for cytotoxicity have been associated with genetic disorders that can be complicated by a hemophagocytic syndrome, namely *RAB27A* mutations have been linked to Griscelli syndrome type 2 (849) and *LYST* mutations have been identified as a cause of Chédiak-Higashi syndrome (850).

X-linked lymphoproliferative syndromes type 1 and 2 are caused by mutations in *SH2D1A* and *XIAP*, respectively. These are two other hereditary immunodeficiencies associated with hemophagocytic syndromes, usually triggered by an Epstein Barr virus infection (851,852). The genetic defects in X-linked lymphoproliferative syndromes cause an aberrant apoptosis of immune cells, leading to prolonged survival of lymphocytes (851,852).

In 2014, two reports described patients with periodic fevers and episodes of hemophagocytic syndrome linked to a gain of function mutation in *NLRC4*, associated with IL1β and Il18 overproduction and pyroptosis (853,854).

In conclusion, since the description of the role of perforin in familial hemophagocytic lymphohistiocytosis, multiple other proteins related to the production, exocytosis and function of cytotoxic granules from $CD8^+$ T lymphocytes and Natural Killer cells have been found to be altered in patients with this disorder (855). This list (Table

8.1) will certainly grow in the following years with new data from exome/genome sequencing studies.

Familial hemophagocytic lymphohistiocytosis is, therefore, a constellation of rare autosomal recessive immune disorders that usually become evident in the first months of life. Patients with *PRF1* null mutations typically present within the first year of life, whereas those with missense mutations and variable degrees of perforin expression have a more variable age of presentation, even into adulthood (732,856–859).

Interestingly, in recent years the clear distinction between primary and secondary hemophagocytic syndromes is becoming more difficult, as new genetic causes are being identified, some of which are associated with less severe phenotypes that occur later in life. Some of these are due to heterozygous or compound heterozygous mutations in genes related to the cytolytic pathway that confer a dominant negative effect (848,860). A digenic inheritance pattern has also been described (861).

Furthermore, preliminary studies suggest that children with sJIA and macrophage activation syndrome have heterozygous mutations or polymorphisms in genes related to the cytolytic pathway (862), including *PRF1* (863) and *UNC13D* (864).

PRF1 was found to be mutated resulting in an amino acid change (Ala91Val) in 20% of sJIA patients from a European population who developed macrophage activation syndrome, 10% of the sJIA patients without known macrophage activation syndrome and 1.0% to 5.5% of the general population (863).

Recently, it was published a report on 500 patients with hemophagocytic syndromes from the Italian registry (865). Interestingly, from the 281 (56%) patients classified as non familial, 43 (15%) had monoallelic mutations in one of the genes associated with familial hemophagocytic lymphohistiocytosis (865). Patients with secondary hemophagocytic syndromes were, therefore, highly enriched for monoallelic mutations in those same genes that produce hemophagocytic lymphohistiocytosis when both alleles are mutated (865).

Disease	Gene	Protein	Protein Function	Notable Clinical Findings
Familial Hemophagocytic Lymphohistiocytosis				
FHL1	Unknown			
FHL2	*PRF1*	PRF1 (Perforin)	Pore forming	
FHL3	*UNC13D*	MUNC13-4	Vesicle priming	Increased incidence of central nervous system involvement; mild bleeding tendency
FHL4	*STX11*	SYNTAXIN 11	Vesicle docking	Mild and recurrent HLH and colitis
FHL5	*STXBP2*	MUNC 18-2	Vesicle membrane fusing	Colitis, bleeding tendency, hearing loss and hypogammablobulinemia
Immunodeficiency syndromes with albinism				
Chédiak-Higashi syndrome	*LYST*	LYST	Vesicle trafficking	Bleeding tendency; pyogenic infections, neuropathy
Griscelli syndrome type II	*RAB27A*	RAB27A	Vesicle fusing	
Hermansky-Pudlak syndrome type II	*AP3B1*	AP3B1	Vesicle trafficking	Bleeding tendency
Other primary immune defects associated with high risk of Epstein-Barr virus infection				
CD27 deficiency	*CD27*	CD27	Lymphocyte costimulation	Combined immunodeficiency
ITK deficiency	*ITK*	ITK	T cell signaling	Hodgkin lymphoma
X-linked lymphoproliferative disorder type I	*SH2D1A*	SAP	Signal transduction and activation of lymphocytes	Hypogammaglobulinemia and lymphoma
X-linked lymphoproliferative disorder type II	*BIRC4*	XIAP	Caspase inhibition	Mild and recurrent HLH and colitis
XMEN	*MAGT1*	MAGT1	T cell activation	Combined immunodeficiency; severe chronic viral infections; lymphoma

Table 8.1 – Mutations associated with hemophagocytic syndromes.

A whole-exome sequencing study was performed in 14 patients with sJIA and macrophage activation syndrome and their parents (866). This study allowed to identify rare protein-altering variants in known familial hemophagocytic lymphohistiocytosis associated genes as well as in new candidate genes (866).

Conceptually, these data are consistent with a two-hit or multi-hit model of hemophagocytic syndromes: genetically susceptible individuals when exposed to certain triggers develop overwhelming inflammation (836). All these data allow us to conclude

that cytotoxic function is not only important for effector immune function, but also for resolution of inflammation (836).

Mouse models helped us to better understand the pathogenesis of hemophagocytic syndromes. Perforin-deficient mice (*Prf*-/-) infected with lymphocytic choriomeningitis virus exhibit the clinical characteristics of human hemophagocytic syndromes, including the development of pancytopenia. Interestingly, *Prf*-/- mice that were also IFNγ deficient were protected from severe anemia (867). Furthermore, IFNγ alone, delivered via osmotic pump, in wild type mice was sufficient to cause hemophagocytosis and anemia, in a dose-dependent manner (867). Transgenic mice that selectively express a dominant-negative mutant IFNγ receptor in macrophage lineage cells and have a near complete block of IFNγ signaling in these cells do not develop hemophagocytosis and are protected from anemia when challenged with IFNγ infusion (867). These data suggest that IFNγ must act directly on macrophages in order for hemophagocytosis to occur *in vivo.* Interestingly, as little as 20% of wild-type perforin-expressing $CD8^+$ T cell chimerism is able to ameliorate the clinical picture in these animals (868). Elimination of antigen-presenting dendritic cells can also protect against the occurrence of hemophagocytic syndrome like manifestations in perforin-deficient mice (869).

Mice deficient in MUNC13-4 and RAB27A also develop a primary hemophagocytic syndrome like phenotype upon infection with the lymphochoriomeningitic virus in an IFNγ dependent manner (870–872).

According to the current model of primary hemophagocytic syndromes, the defective natural killer cells and cytotoxic T lymphocytes fail to kill infected cells, causing antigen persistence and sustained activation and proliferation of T lymphocytes. Moreover, abnormal cytotoxic cells fail to send appropriate apoptotic signals for removal of activated T lymphocytes and macrophages, causing persistent expansion of these cells and increased secretion of proinflammatory cytokines, including IFNγ, TNF, IL6, Il10, Il12, Il16 and IL18 (873–876). As a result of continuous stimulation with proinflammatory cytokines, mainly IFNγ, macrophages will become hemophagocytic (695,877).

A recent case-control study highlighted the importance of another cytokine: IL18 for the pathogenesis of macrophage activation syndrome (878). Levels of IL18 were measured in the serum of 76 patients with active sJIA, of whom 15 patients developed

macrophage activation syndrome (878). During the macrophage activation syndrome, IL18 levels were significantly increased (878). Furthermore, patients with active sJIA who developed macrophage activation syndrome during the course of the disease had a significantly higher level of IL18 when compared with those who did not develop macrophage activation syndrome (878).

Recently, the role of another cytokine, IL33, was highlighted in a mouse model (879). Il33 is an alarmin that activates an inflammatory response in the context of tissue damage. It was shown that perforin-deficient mice (*Prf*-/-) infected with lymphocytic choriomeningitis virus had upregulation of IL33 and its receptor - ST2 (879). ST2 signaling promoted $CD8^+$ and $CD4^+$ T cell activation and production of IFNγ, leading to lethal inflammation (879). Blockade of ST2 improved survival and reduced the severity of the illness and the IFNγ levels (879).

Another mouse model uses transgenic mice that carry a human *IL6* transgene and constitutively produce high levels of IL6 (880). These transgenic mice, when stimulated with TLR ligands, have an increased fatality rate, when compared to wild type mice, indicating that prolonged exposure to IL6 leads to an exaggerated inflammatory response to TLR ligands (880). These data lead to speculation regarding the possible pathogenesis of macrophage activation syndrome in the setting of sJIA. High circulating IL6 levels, typical of this disease, may predispose to hyperresponsiveness to infections (880). Furthermore, a recent study showed that, in both mice and humans, IL6 down-modulated the cytotoxic activity of natural killer cells, reducing perforin and granzyme B levels in the absence of altered granule exocytosis (881). This is a new interesting connection between secondary and primary hemophagocytic syndromes.

A different murine model of macrophage activation system induced by repeated stimulation of TLR9 has contributed to a better understanding of this condition (882). This mouse model is important since it is not dependent on a genetically mediated cytotoxic defect or a viral infection, resembling more closely what happens in Humans. As previously discussed in Chapter 2, TLR9 is a receptor for DNA containing unmethylated CpG sequence motifs. There are multiple studies connecting TLR9 hyperactivation and macrophage activation syndrome. In sJIA patients there is a gene signature consistent with chronic TLR/IL1β signaling (883) and polymorphisms of IRF5, a molecule that participates in TLR9 signaling, have been associated with macrophage activation

syndrome in these patients (884). TLR9 also seems to participate in SLE pathogenesis, since the number of TLR9-expressing B cells and monocytes is higher in patients with active SLE (266). Furthermore, the number of TLR9 expressing-B cells correlated with anti-dsDNA antibodies levels (266). Epstein Barr virus infection, one of the most common triggers of secondary hemophagocytic syndromes, is a DNA virus that triggers TLR9 (885). The murine model of TLR9 stimulation replicates, therefore, the environment that allows macrophage activation syndrome to occur in the genetically predisposed host.

Mice submitted to TLR9 stimulation develop features similar to macrophage activation syndrome (882). The disease in this model is fulminant, if there is a concordant IL10 blockade (882). Interestingly, polymorphisms of *IL10* associated with decreased function are associated with sJIA, showing that IL10 may play a protective role (886,887). Unlike what is seen in mouse models for primary hemophagocytic syndromes, in the TLR9 hyperactivation model, depletion experiments revealed that T cells, Natural Killer T cells and B cells were not major pathogenic effector cells. In addition, fulminant hemophagocytic syndrome like manifestations occurred in the absence of IFNγ, Il12, TNF, INFα and INFβ, demonstrating that this condition is driven by a complex cytokine milieu, being independent of any single cytokine (888).

This mouse model also helped us understand the link between TLR9-induced hyperinflammation and decreased B cell lymphopoiesis (889). It is, therefore, possible to speculate that B cell autoimmunity in SLE might in part arise from dysfunctional B cell lymphopoiesis due to TLR stimulation (889).

Another important contribution from this model was the dissociation between anemia and hemophagocytosis. Repeatedly CpG-treated wild type mice exhibited anemia in the absence of hemophagocytosis, while CpG-treated IFNγ -/- mice with IL-10 blockade had hemophagocytosis, without anemia (882,888). These data suggest that IFNγ mediated dyserythropoiesis, not hemophagocytosis, is the dominant mechanism of macrophage activation syndrome associated anemia (888).

Even though in the past the presence of hemophagocytosis was correlated with the development of anemia and with disease activity, there is no direct demonstration of causality, namely that hemophagocytes are directly pathogenic. On the contrary, recent studies suggest that hemophagocytes may have a regulatory function. The erythrophagocytosis may provide a substrate for the production of heme oxygenase-1,

which is known to be protective in sepsis by limiting the inflammatory response (712,890).

CD 163, which is highly expressed in hemophagocytes (721), is a marker strongly associated with regulatory M2 differentiation.

Interestingly, it was found that hemophagocytes are an important source of IL10 (891). When hemophagocytosis is blocked or hemophagocytes are incapable of producing IL10, viral induced mortality is increased (891). Hemophagocytes may constitute, therefore, a negative feedback machinery with regulatory functions rather than being pathogenic contributors of the disease as was previously thought (891).

Hemophagocytic syndromes are heterogeneous conditions linked by the common finding of overwhelming inflammation. Data from basic research studies led to a multi-hit model, where genetically susceptible individuals develop uncontrolled inflammation when exposed to certain triggers. This happens not only in the cases of familial hemophagocytic lymphohistiocytosis, who have inherit defects on cytotoxicity, and develop a hemophagocytic syndrome after an infection trigger, as well as in secondary forms of hemophagocytic syndromes. In sJIA, for instance, the continuous exposure to IL6 is associated with hyperresponsiveness to infectious triggers and induced defects on cytotoxicity.

In conclusion, in the last couple of years a profound change occurred in the clinic approach of hemophagocytic syndromes. New criteria were established and new treatment strategies were tested. We are living in an exciting new era for diagnosis and treatment of these conditions that hopefully will improve the survival of these patients. There are still, however, pressing questions to be answered. The function of hemophagocytes is certainly one of them.

Morphologically, hemophagocytes are macrophages that have engulfed other hematopoietic cells. Macrophages reside in organs throughout the body where they are involved in diverse functions including pathogen sensing, pro- and anti-inflammatory immune responses, and wound healing. Recent evidence demonstrates that environmental factors, particularly the cytokine milieu, determine the macrophage activation status in a continuum ranging from M1 to M2 (892–896). M1 macrophages, driven by IFNγ and LPS, typically acquire pro-inflammatory properties and are associated with tissue damage, whereas M2 macrophages have more heterogeneous stimuli and

functions, being associated with immunoregulation, tissue remodeling and fibrosis (893,896). Thus, macrophage functions can be fluid and varied, proinflammatory or anti-inflammatory, depending on the mix of signals they receive. New studies are necessary to better understand the complex pathophysiology of hemophagocytosis, particularly to help us determine whether hemophagocytes contribute to the disease progression or whether they appear as a consequence of its severity, being responsible for an anti-inflammatory effect.

8.2 GOALS

The goal of this study was to analyze the transcriptional program of morphologically identified murine hemophagocytes and to describe the surface markers of Human hemophagocytes. The ultimate aim was to better understand the role of hemophagocytes.

8.3 METHODS

8.3.1 Isolation of murine hemophagocytes

Fulminant macrophage activation syndrome was induced with repeated TLR9-stimulation, via CpG administration, and IL10 receptor blockade as previously described (888). Splenic touch preps were made on nuclease free polyethylene naphthalate membrane-coated slides (Zeiss) and immediately Wright-Giemsa stained. A pediatric hematopathologist with expertise in hemophagocytic syndromes morphologically identified hemophagocytes from TLR9-stimulated, IL10 receptor-blocked mice or resting macrophages from saline-treated mice. From each mouse, twenty cells were captured, using the Zeiss/P.A.L.M. laser microdissection microscope, isolated, pooled, and processed in aggregate.

8.3.2 RNA isolation and microarray

cDNA libraries were generated from the RNA of pooled, microdissected cells using the WT-Ovation One-Direct Amplification System (NuGen Technologies) according to

manufacturer's instructions. Fragmented cDNA was then hybridized to Affymetrix Mouse Gene ST 1.0 microarrays, washed, stained and scanned with the Affymetrix Scanner 3000 7G.

8.3.3 Transcriptional analysis

Affymetrix Expression Console software was used to perform quality control, excluding two chips from each group and leaving four biological replicates per group for analysis. Microarrays were preprocessed using robust multiarray analysis (897)(Gene Expression Omnibus accession number GSE47430). Probe sets lacking gene symbol annotation or with a mean $\log_2$ intensity less than five among hemophagocytes samples were filtered out. Log_2 transformed expression data were analyzed using the R statistical computing language. Differentially expressed genes were defined as those with at least 1.5-fold difference in expression in hemophagocytes versus resting macrophages. Differentially expressed genes were tested for statistical significance using Student's t-test with multiple testing correction using false discovery rate (FDR). Functional enrichment analysis among differentially expressed genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (898). Gene ontology terms for biological pathways, molecular function and cellular components were tested, as were Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways. Genes passing filtering criteria were used as the background gene list in the enrichment analysis.

8.3.4 Gene set enrichment analysis

Gene sets were identified by searching the Molecular Signatures Database (MSigDB) for the term "inflammation" (9). Gene sets with substantial overlap were excluded. Initially, 17 gene sets were included in this analysis, but the "Phagocytosis" gene set was excluded as too few probes were included in our dataset. MOgene expression data was translated into Human Genome Organization (HUGO) gene symbols before comparison with selected gene sets, as per gene set enrichment analysis protocol. Gene sets were analyzed using gene set permutation, and prior to analysis we chose a threshold for significant enrichment of: nominal p-value ≤0.05 and FDR ≤5% (899).

8.3.5 Patient selection

We performed an unbiased, retrospective search of the pathology database of The Children's Hospital of Philadelphia for bone marrow specimens performed between 1998 and 2011 whose clinical report documented excessive hemophagocytosis. Thirty-seven samples from 34 patients were identified and verified as having excess hemophagocytosis. These samples had been decalcified, fixed in acetic acid-zinc-formalin (AZF), and paraffin-embedded per institutional protocol. Chart review identified clinical information related to each sample. This study was approved by the institutional review board of The Children's Hospital of Philadelphia.

8.3.6 Selection of macrophage markers

Monocytes from healthy donors were cultured *in vitro* and stimulated for three days with IFNγ or IL4. Their phenotype was analyzed by flow cytometry and immunohistochemistry. We also evaluated these receptors by immunohistochemistry in bowel samples from Crohn disease patients (an M1 model) and peritoneum from a patient with *Schistosoma mansoni* infection (an M2 model).

8.3.7 Imunohistochemistry of human hemophagocytes

Immunohistochemistry was performed for CD64 (1:25; clone 10.1, Abcam), CD163 (900) (1:200; clone 10D6; Vector Laboratories) and CD206 (1:500; clone 5C11, Abnova/Novus Biologicals). Heat antigen retrieval was utilized for CD64, at pH 6, and CD163, at pH 9. Avidin-biotin complex signal amplification was used for CD206 and a polymeric–based method for CD64 and CD163. Detection was performed with horseradish peroxidase and 3,3' Diaminobenzidine (DAB) chromogen, and the sections were counterstained with hematoxylin. Adequate positive and negative staining controls were performed in AZF fixed tonsils. Patient sections were evaluated for the presence or absence of immunostained HPCs by a blinded hematopathologist with expertise in hemophagocytic diseases.

8.4 RESULTS

8.4.1 Genes associated with macrophage activation and regulation of inflammation are among the most upregulated in murine TLR9-induced hemophagocytes

Forty-five genes met our criteria for differential expression (Table 8.2).

No genes with decreased expression in hemophagocytes were found to be significant. Genes encoding the ribosomal subunits Rps20 and Rpl35a were among the most highly upregulated, suggesting regulation of global protein synthesis by hemophagocytes. Consistent with this, a functional enrichment analysis of all differential expressed genes suggested that "translation" was a significant biological function of hemophagocytes (Gene Ontology biological process annotation, Benjamini-corrected p-value=0.002). We further found the KEGG "Ribosome" pathway was significantly enriched (Benjamini-corrected p-value=1.6×10^{-7}).

Another group of upregulated genes were related to cellular energetics. Two genes encoding subunits of the mitochondrial respiratory protein complex cytochrome c oxidase (COX6C, COX6A1) were upregulated, as well as the glycolytic enzyme GAPDH. Accordingly, the KEGG "Oxidative Phosphorylation" pathway was significantly enriched in genes differentially expressed by hemophagocytes (Benjamini-corrected p value=4.2×10^{4}). Thus, these data suggest TLR9-induced hemophagocytes are acting to meet demands for energy and protein synthesis.

Genes associated with macrophage stimulation and activation were also among the most differentially expressed. β2-microglobulin and IFITM2 are upregulated with anti-viral responses, while SAA3 and calcyclin are parts of the acute phase response.

Upregulation of the ferritin light chain (FTL) gene by hemophagocytes is consistent with the hyperferritinemia characteristic of hemophagocytic syndromes. Finally, the homologues Thymosin β10 and β4 may play regenerative and anti-inflammatory roles in macrophages.

Gene	Protein Product	Fold Change	P-value	FDR
Rps20	Ribosomal Protein S20	22.6	0.0063	0.146
β2m	β-2 microglobulin	12.5	0.0292	0.206
*Rpl35a**	Ribosomal Protein L35A	8.5	4.29e-4	0.055
Saa3	Serum Amyloid A 3	7.9	0.0014	0.055
Ifitm2	IFN-inducible transmembrane protein 3	6.7	0.0445	0.210
Cox6c	Cytochrome c oxidase, subunit Vic	6.2	0.0238	0.174
*Ftl**	Ferritin light chain	5.8	0.0028	0.055
Tmsb10	Thymosin β10	4.9	0.0430	0.210
*Gapdh**	Glyceraldehyde 3 phosphate dehydrogenase	4.9	0.0019	0.055
Cox6a1	Cytochrome c oxidase subunit VIa, polypeptide 1	4.2	0.0388	0.210
Usmg5	Upregulated during skeletal muscle growth 5	4.2	0.0412	0.210
Tmsb4x	Thymosin β4	4.1	0.0427	0.210
S100a6	S100 calcium binding protein A6 (calcyclin)	4.1	6.27e-4	0.055
*Target identified by more than one probe.				

Table 8.2 – Highly differentially expressed genes in TLR9-induced hemophagocytes versus resting macrophages.

8.4.2 Gene set enrichment analysis suggests alternative polarization of murine TLR9-induced hemophagocytes

To refine our analysis of differential gene expression, we tested for upregulation of gene sets associated with a variety of relevant transcriptional programs in hemophagocytes. Only three gene sets fulfilled our criteria for significance: proteasomal degradation, nod-like receptor signaling, and the set of genes upregulated by M2 versus M1 polarized macrophages (Table 8.3) (899). Notably, the set of genes upregulated in M1 versus M2 macrophages was not found to be significantly enriched in hemophagocytes.

Gene Set	Size	Normalized enrichment score	Nominal p	FDR
Proteasome	43	2.42	< 0.001	< 0.001
Upregulated in M2 vs M1	73	1.85	< 0.001	0.009
NOD-like Receptor Signaling	54	1.74	0.002	0.022
Innate Immunity Signaling	97	1.49	0.015	0.133
Actin Regulation	195	1.48	0.007	0.116
Cytosolic DNA Sensing	46	1.48	0.058	0.101
TLR Signaling	90	1.41	0.044	0.127
Upregulated in M1 vs M2	76	1.32	0.080	0.188
Endocytosis	160	1.23	0.12	0.283
TLR3 Cascade	56	1.04	0.391	0.545
TLR4 Cascade	25	0.93	0.556	0.717
IL10 Pathway	17	0.84	0.663	0.839
Integrin Pathway	37	0.61	0.963	1
TLR9 Cascade	20	0.54	0.969	0.989
Inflammatory Pathway	25	-0.95	0.547	1
IL1R Pathway	30	-0.67	0.921	0.946

Table 8.3 – Gene set enrichment analysis of TLR9-induced hemophagocytes versus resting macrophages.

8.4.3 CD64, CD163 and CD206 are markers of macrophage activation status

In humans, we identified, through immunohistochemistry and flow cytometry, surface markers of macrophage activation status. We identified CD64 as a M1 marker and CD206 as a M2 marker by immunohistochemistry (Figure 8.1 and Figure 8.2). CD64 was increased when human monocytes were exposed to IFNγ and it was overexpressed in the bowel of patients with Chron disease and not expressed in the hernia sac of a patient infected with a parasite (Figure 8.2 and Figure 8.3).

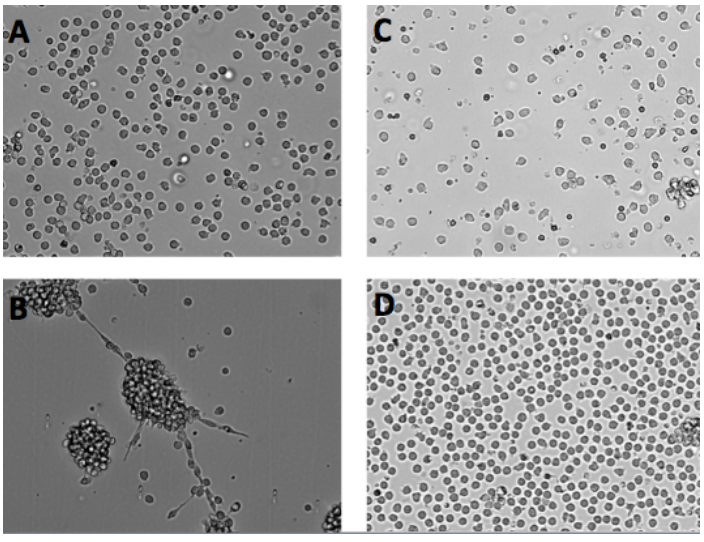


Figure 8.1 – Human monocytes from healthy donors exposed during three days to different cytokines. A. No cytokines. **B.** IFNγ. **C.** IL4. **D.** IFNα. All images were captured at 200x.

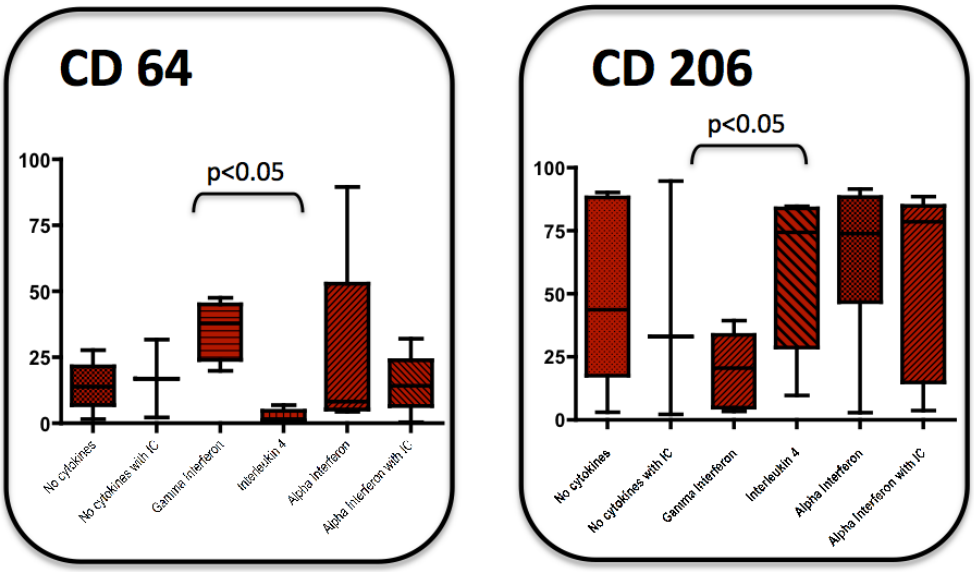


Figure 8.2 – Results from flow cytometry studies performed on human monocytes exposed to different cytokines. The charts show the percentage of cells that express the surface marker. IC- Immune complexes.

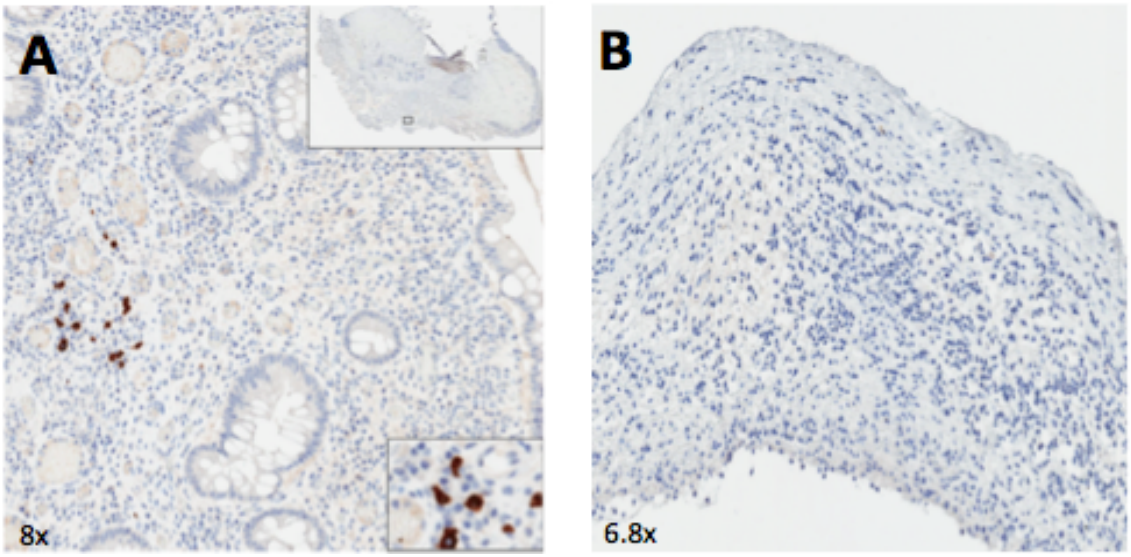


Figure 8.3 – Immunohistochemistry for CD64 in two different models of macrophage activation. **A.** Bowel from a patient with Crohn disease. **B.** Hernial sac from a patient infected with *Schistosoma mansoni.*

CD206 was increased when human monocytes were exposed to IL4 (Figure 8.2) and was overexpressed in the hernia sac of a patient infected with *Schistosoma mansoni*, but not in Chron Disease (Figure 8.4).

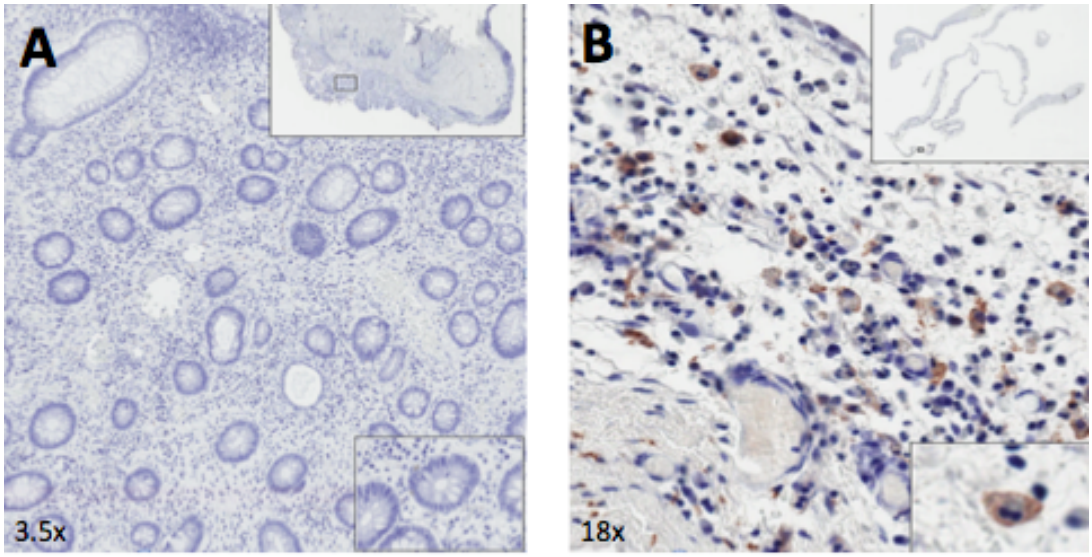


Figure 8.4 – Immunohistochemistry for CD206 in two different models of macrophage activation. A. Bowel from a patient with Crohn disease. **B.** Hernial sac from a patient infected with *Schistosoma mansoni.*

CD163 was also expressed in the M2 model, namely in the hernia sac of a patient infected with a parasite (Figure 8.5).

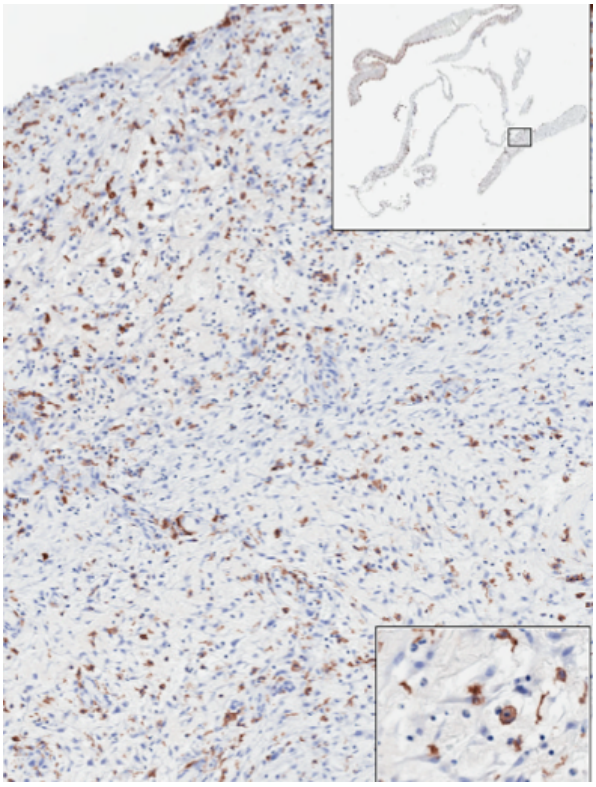


Figure 8.5 – Immunohistochemistry for CD163 in the hernial sac of a patient infected with *Schistosoma mansoni.*

8.4.4 Hemophagocytes from a diverse human cohort uniformly express CD163, and rarely express CD206 or CD64

We identified a longitudinal cohort of human bone marrow samples that on biopsy or aspirate showed excess hemophagocytosis regardless of diagnosis. These samples represented hematologic malignancies (lymphoma-1, Langerhans-cell histiocytosis-3, and post-transplant lymphoproliferative disease-3), infections (Epstein Barr virus-6, bacterial-2, parvoviral-1, and fungal-1), immunodeficiencies (paroxysmal nocturnal hemoglobinuria-1 and MUNC13-4 deficiency-1), rheumatic diseases (systemic and other forms of JIA-9 and dermatomyositis-1) and idiopathic causes of hemophagocytosis-9).

Regardless of disease of origin or treatment stage, bone marrow samples demonstrated positive staining of hemophagocytes for CD163 in all tested samples (Figure 8.6). CD163 is a scavenger receptor that binds and internalizes hemoglobin–haptoglobin complexes, which then activate hemeoxygenase 1 and induce the synthesis of ferritin (901).

Five samples, representing diverse diseases, stained positively for CD206, a mannose-receptor and marker of alternative or M2-macrophage activation (Figure 8.6) (902).

Two samples, both from patients with systemic infections, showed positive staining of hemophagocytes for CD64, the Fcγ-receptor 1 and a marker of classical/M1-macrophage activation (Figure 8.6) (902). No samples demonstrated positive staining of hemophagocytes for both CD64 and CD206.

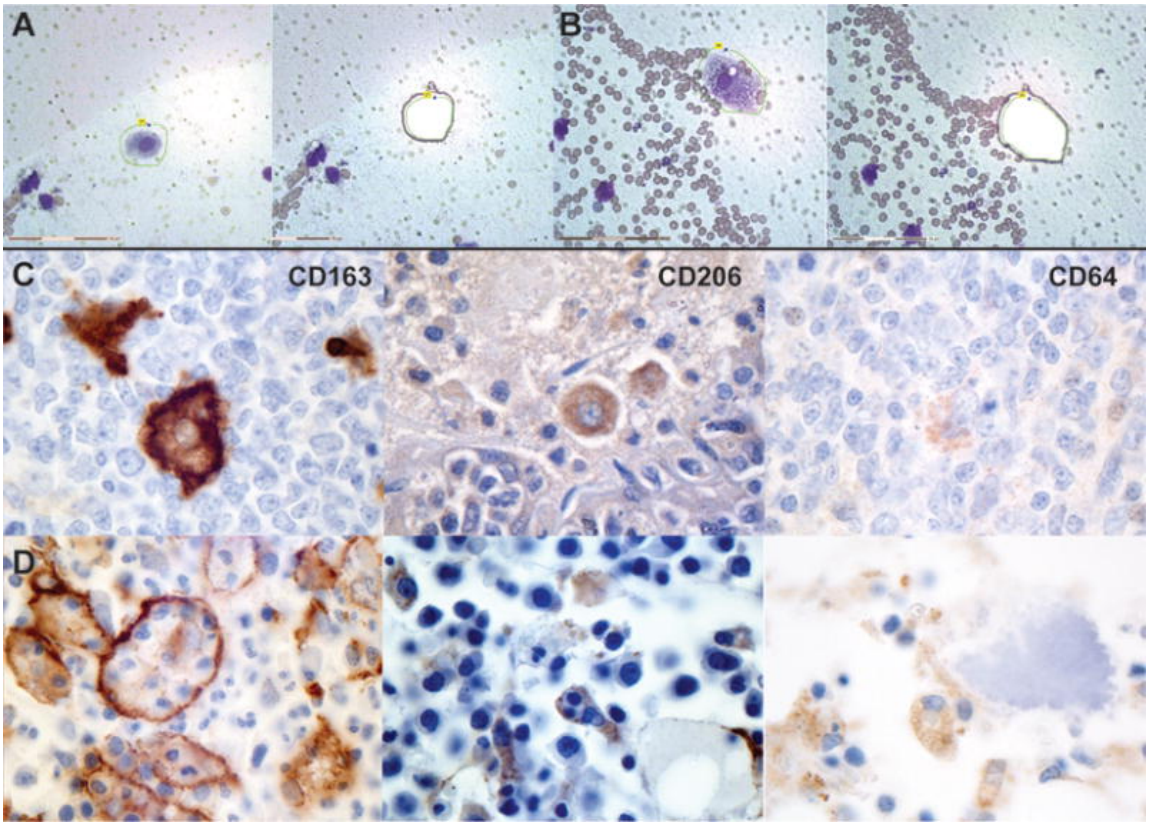


Figure 8.6 – Murine and human hemophagocytes. **A.** Laser capture microdissection of a resting murine splenic macrophage. **B.** Laser capture microdissection of a murine hemophagocyte. **C.** Positive controls for immunohistochemical stains in tonsil tissue from patients without hemophagocytic disease. **D.** Bone marrow biopsies from representative patients with hemophagocytic disease immunostained for CD163, CD206, or CD64. All images are 100X magnification and slides are counterstained with hematoxylin.

8.5 DISCUSSION

Hemophagocytes are enigmatic cells appearing in a variety of inflammatory contexts. Their presence often influences important medical decisions regarding evaluation and treatment. However, our poor understanding of the function of these cells makes the interpretation of their presence, or absence, confusing. Our analysis of the transcriptional program of morphologically – identified murine TLR9-induced hemophagocytes suggests that they are alternatively activated.

The “fulminant TLR9-macrophage activation syndrome” model was chosen due to the homogeneity of hemophagocytosis, the absence of confounding by infection or genetic alteration, and the fact that mice treated with TLR9 stimulation or IL10R blockade alone fail to develop hemophagocytes (888).

Examination of the genes most upregulated by hemophagocytes showed, among others, induction of *Ftl* (ferritin light-chain) and *Saa3*. Astoundingly high ferritin levels are characteristic of hemophagocytic syndromes, and ferritin has been associated with anti-inflammatory transcripts in sJIA (883). Both ferritin and SAA3 production have been associated with anti-inflammatory activity in macrophages (721,903).

We also found that cytochrome oxidase genes, oxidative phosphorylation pathways, and the canonically glycolytic enzyme GAPDH were upregulated in hemophagocytes. While this might suggest induction of both glycolysis and oxidative phosphorylation, GAPDH is increasingly associated with a variety of non-glycolytic functions including inhibition of cytokine translation (904).

The M2 versus M1 gene set was generated by evaluating genes upregulated by *in vivo* skewed M2 macrophages compared to M1 skewed macrophages (899). We found that this M2-associated gene set, but not the M1 gene set, was strongly associated with genes differentially upregulated by hemophagocytes, supporting alternative activation. The other enriched gene sets, nod-like receptor signaling and proteasome activity, may be associated with scavenger receptor activity (905) or the degrading requirements of hemophagocytosis, respectively.

RNA from laser-captured macrophages served as the basis for our microarrays. Our gene expression analyses were limited by the necessary pooling of individual cells' RNA from any single mouse, and by small input amounts of RNA. Thus, the potential for M1 versus M2 heterogeneity exists within mice and even within individual cells. Additionally, these results have not been validated by quantitation of specific targets. The limited starting material made protein quantitation impossible. Additionally, the process of amplifying small input RNA has limited our ability to quantitate at the RNA level: the RNA fragments that result from this process, while of ideal size for microarray, were of insufficient length of conventional quantitative PCR. However, our analyses were able to detect changes associated with macrophage activation. Specifically, our transcriptomic data suggest that the dominant program of murine hemophagocytes induced by TLR9 stimulation and IL10R blockade is skewed towards alternative activation. Accordingly, recent data support the notion that TLR9-driven hemophagocytes arise independently of a number of pro-inflammatory cytokines including IFNγ (888) and may exert anti-inflammatory effects (891).

Immunohistochemistry for selected markers in human bone marrow samples supported CD163 expression by hemophagocytes in a cohort that, to our knowledge, is unique in terms of its breadth of diagnoses. The association of CD163 with alternative activation is well-established (901). Future studies should attempt to validate further the findings of TLR9-stimulated murine hemophagocytes in human samples.

8.6 CONCLUSIONS

We have shown transcriptional evidence for alternative activation of murine hemophagocytes from an infection-free model, as well as immunohistochemical evidence that human hemophagocytes from a diversity of sources express CD163. These data support the notion of hemophagocytes as alternatively-activated macrophages occurring as a common response to systemic inflammation.

In hemophagocytic syndromes there is likely heterogeneity in macrophage function, and therapeutically depleting all macrophages could actually be detrimental.

There has been, therefore, a complete change in the way we interpret the role of hemophagocytes in these syndromes. Not only they are not necessary for the anemia, they may even have a regulatory, anti-inflammatory, function. Perhaps a new nomenclature should be used to define these syndromes, considering that hemophagocytosis is no longer the most important step on its pathogenesis. Further studies are warranted to better understand the induction of hemophagocytosis, the precise functions of hemophagocytes, and the roles these cells play in regulating immunopathology.

Please refer to **Appendix A.4** for the paper ***Uma Nova Era no Diagnóstico e no Tratamento da Síndrome Hemofagocítica***, which was published in the journal *Acta Pediátrica Portuguesa*. In this paper it is reviewed the diagnosis and treatment of the different types of hemophagocytic syndromes.

The **Appendix A.5** includes the paper ***Alternative Activation of Laser-Captured Murine Hemophagocytes***, which was published in the journal *Arthritis and Rheumatology* and gathers the original data from the study of the phenotype of hemophagocytes.

CHAPTER 9

The Transcriptome of Systemic Lupus Erythematosus

9.1 INTRODUCTION

As previously described in Chapter 2, monocytes are a critical cell in SLE. They are implicated in renal damage, which is the major cause of morbidity in SLE, and in atherosclerosis, which is the major cause of mortality in SLE (353,906–908). Monocytes are therefore central to the disease process but are also of interest because they respond to environmental stimuli, alter their function accordingly, and reflect that information back to other immunologically competent cells. They offer the additional advantage of representing a relatively homogeneous population.

There remains much that is unknown about the pathogenesis of SLE, particularly at the level of nucleic acid accumulation and dysregulated gene expression. Monocytes are, thus, a pivotal cell to perform these studies in SLE.

9.2 GOALS

The main goal of this study is to characterize the transcriptome of monocytes of SLE patients by next generation sequencing of transcripts.

9.3 METHODS

9.3.1 Cell purification

Investigators at Johns Hopkins University School of Medicine obtained written informed consent and HIPAA Authorization of study subjects for all SLE samples. The IRB at Johns Hopkins reviewed and approved the study of SLE patients. The use of the anomyzed Red Cross samples was approved by the Red Cross IRB. Control samples were also obtained from The Center For Aids Research, which supplies blood samples on a fee for service basis. They have obtained consent for the use of the samples and their protocols were approved by the University of Pennsylvania Institutional Review Board.

Primary human monocytes were purified using elutriation and adherence from eight healthy controls and nine SLE patients, as previously described (549–551). The

purity of monocytes was more than 90% by flow cytometry for CD14 staining. All controls and patients were female, with an average of approximately 40 years of age. All SLE patients' disease activity was mild to moderate and no one was on high-level immune suppression (Table 9.1). All subjects were provided informed consent, except the Red Cross serum samples which were provided as anomyzed discarded samples.

Patient	SLEDAI	Organ involvement	Current Medications	Autoantibodies
1	7	Skin, arthritis, vasculitis, serositis	Hydroxychloroquine Prednisone ≤10mg/d	ANA, Anti-dsDNA, Anti-RNP, Anti-SM, ACL
2	0	Skin, arthritis	Hydroxychloroquine NSAID	ANA, Anti-dsDNA
3	0	Arthritis	Hydroxychloroquine NSAID	ANA, Anti-dsDNA
4	0	Skin, arthritis, serositis	Hydroxychloroquine Prednisone ≤10mg/d Azathioprine NSAID	ANA, Anti-dsDNA
5	2	Skin, arthritis, serositis, vasculitis	Hydroxychloroquine Prednisone ≤10mg/d Azathioprine NSAID	ANA, Anti-dsDNA, ACL
6	0	Skin	Hydroxychloroquine	ANA, Anti-SSA, Anti-SSB, ACL
7	0	Arthritis, renal, central nervous system	Hydroxychloroquine Azathioprine	ANA, ACL
8	2	Arthritis	NSAID	ANA, Anti-dsDNA, ACL
9	4	Skin, serositis, central nervous system	Hydroxychloroquine Prednisone ≤10mg/d NSAID	ANA

Table 9.1 – Clinical characteristics of the patients studied. ACL- Anticardiolipin antibodies; ANA – Antinuclear antibodies; Anti-RNP – Antiribonucleoprotein antibodies; Anti-SM – AntiSmith antibodies; NSAID - Nonsteroidal anti-inflammatory drugs.

9.3.2 RNA isolation and library preparation

Total RNA was isolated from 2-3 million primary monocytes using the Qiagen RNeasy kit and DNA was removed by on-column DNase digestion (Qiagen, Valencia, CA). This method recovers predominantly RNA species >200bp. One microgram of total RNA was used to prepare the library with the SOLiD™ whole transcriptome analysis kit (Applied Biosystems). The procedure followed the instructions of the manufacturer. For miRNA validation, we used miRNeasy kit (Qiagen). The OD 260/280 ratio ranged from 1.80–1.98. The RNA quality and counts were not different between patients and controls.

9.3.3 RNA abundance validation

qRT-PCR was used to define quantitative differences in RNA abundance. The Clontech Advantage RT for PCR kit (Clontech) was used to generate cDNA. Gene expression was detected by RT-PCR using the TaqMan 9600. Transcript levels were normalized to the 18S or βactin signal. Mature miRNAs were detected with Taqman miRNA assays. Relative quantification was performed using spiked *Caenorhabditis elegans* miRNA-238 as an exogenous control (Qiagen Syn-cel-miR-238-3p miScript miRNA Mimic). Commercially available primers were purchased from Applied Biosystems for: cel-miR-238 (248 primer for isoform cel-miR-238-3p - MIMAT0000293); hsa-miR-212 (515 primer for isoform hsa-miR-212-3p - MIMAT0000269 and 461768_mat for isoform hsa-miR-212-5p - MIMAT0022695); and *CCR2* (Hs00174150 primer for *CCR2* isoform NM_001123041.2 and Hs00704702_s1* for *CCR2* isoform NM_001123396.1) and from Qiagen for *RND3* (QT00002744), *TSLP* (QT01670809), *RGPD1* (QT01678425), *CD177* (QT02452849), *TUBB1* (QT00049574), and *ITG-B* (QT01003121). Novel loci were detected with custom primers using SYBR green. The Mann Whitney U test was used to analyze the differences between SLE and controls.

9.3.4 Endotoxin analyses

Monomac 6 cells and primary monocytes from healthy donors were stimulated with 100U/ml IFNα2 (PBL Biomedical Laboratories), 10ng/ml IFNγ (R&D Systems), 10ng/ml TNF (Sigma) for 16 hours, or 1mg/ml of LPS for two hours. SB203580 was used as a p38-MAPK inhibitor by pretreating the cells for 30 minutes at a concentration of 10mM. SP600125 (Calbiochem) was used as a JNK inhibitor at a concentration of 10 µM and U0126 (Cell signaling) was used as an ERK inhibitor at a concentration of 10 µM. Cells were harvested after stimulation and RNA was prepared as described above.

To measure circulating endotoxin, serum samples from 99 SLE patients and 112 Red Cross blood donors were analyzed using the Limulus assay (Thermo Scientific). The Wilcoxon method was used to compare the levels across groups.

9.3.5 Bioinformatics

We used the Tophat-Cufflinks pipeline and further refinements to assemble the monocyte transcriptome and detect novel loci and isoforms, followed by mapping short reads to a collection of reference RNA sequences, including isoforms of coding genes, small RNAs, long non-coding RNAs (lncRNAs), and repetitive elements. The number of reads mapped to each transcript was used for evaluating differential expression between control and SLE samples. Data has been submitted to GEO as GSE53419. Loess adjustment was applied by assuming an equal amount of total RNA between samples. The global average of patient-control difference was very close to zero (0.4% higher in SLE) after the adjustment.

Statistical analysis of differential expression was performed within R environment using Bioconductor packages. Genes without at least three read counts in at least three samples were excluded. Gene-gene correlation analysis was performed by the following steps: calculate the correlation coefficients between genes in control and SLE groups separately; convert correlation coefficients to z scores using Fisher's transformation; take the average of z scores, and convert the average z score back to a single combined correlation coefficient r. Enrichment studies utilized DAVID which reports adjusted p values based on the Benjamini Hochberg algorithm (909).

9.4 RESULTS

9.4.1 Transcriptome characterization

Monocyte transcriptome of nine female SLE patients and eight female healthy controls were studied using RNA-seq. Our first goal in this study was to reconstruct the human monocyte transcriptome using the entire dataset. The Tophat-Cufflinks pipeline reported four major classes of transcripts (Figure 9.1), including 10,313 isoforms of known genes and 10,200 transcribed loci not included in the RefSeq annotation. The novel isoforms had a more complex structure. On the other hand, the novel loci were shorter on average than known genes and over 97% of them included only one exon. To further define the monocyte transcriptome, we counted the total number of reads solely aligned to each of the four major RNA classes: coding RNAs, lncRNAs, small RNAs, and repetitive elements. Ribosomal RNAs were excluded from this summary.

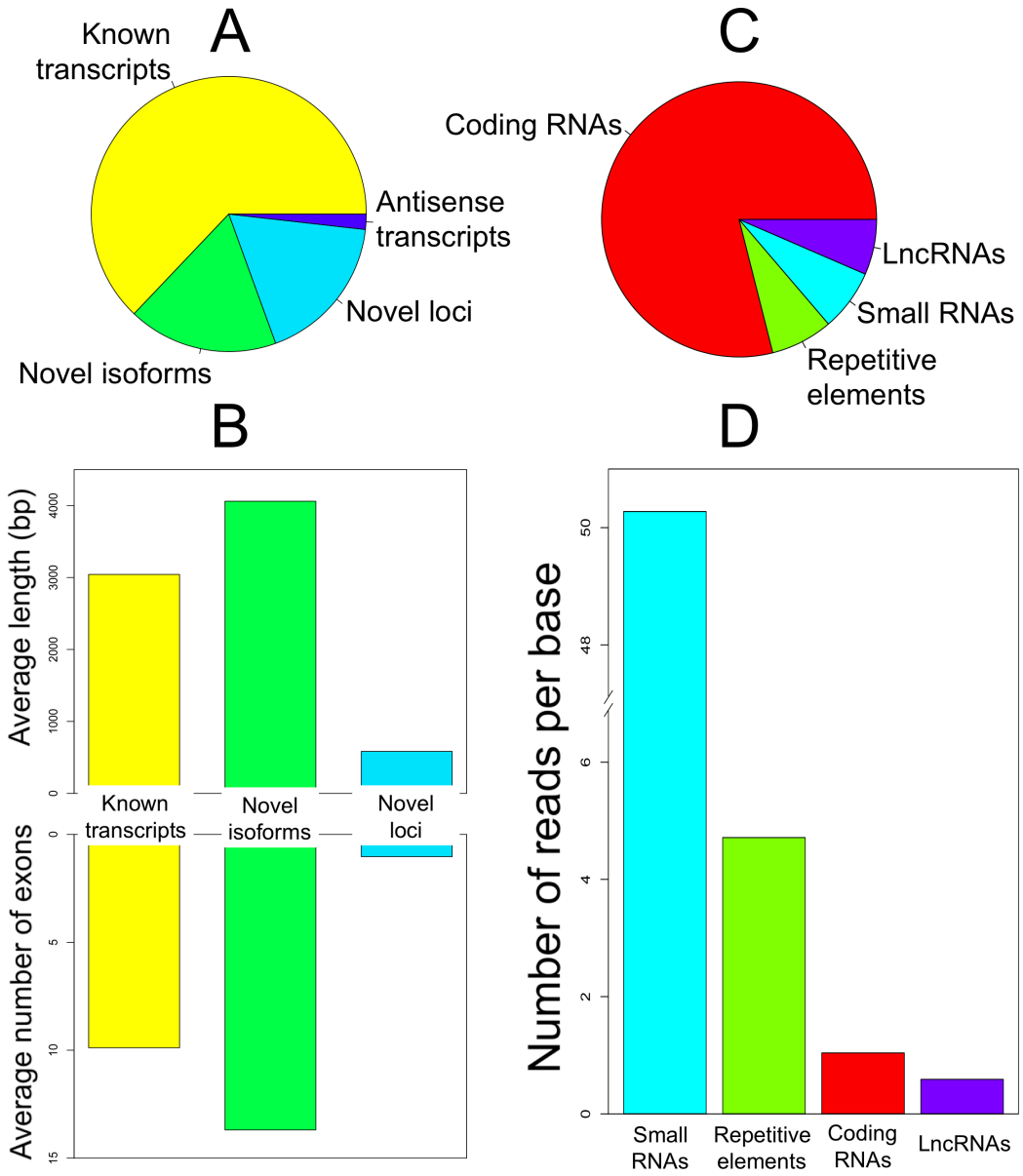


Figure 9.1 – Overall transcriptome characteristics. A. The Tophat-Cufflinks pipeline identified four major classes of transcripts from 17 RNA-seq libraries. RefSeq genes constituted the majority of the transcripts. **B.** Novel isoforms and loci were different from known transcripts in terms of average length and numbers of exons on average. **C.** Coding RNA was the most abundant RNA species (except ribosomal RNA) in monocytes based on the count of RNA-seq reads. Non-coding RNA collectively accounted for approximately 20% of total RNA. **D.** Small RNAs had the highest expression level on average after adjusting read counts for the total length of RNA classes.

Close to 80% of the non-rRNA reads were mapped to coding RNAs while the other three classes evenly shared the remaining reads (Figure 9.1C). Small RNAs had the highest molar concentration after read counts were adjusted by the total length of the RNA classes, followed by repetitive elements, coding RNAs, and lncRNAs (Figure 9.1D). These data were comparable to the distribution seen in other systems (910).

The classifications of transcripts were refined. We identified 4,000 high-novelty isoforms that were not present in seven additional annotated databases and included at least one unknown exon-exon junction, as well as 2,271 high-novelty loci that had no overlap with either exons or introns of any previously reported transcripts. We then defined 1,327 high-confidence isoforms, having at least 10 uniquely mapped reads not mapped to any other genes or isoforms of the same gene in at least six samples, and 3,725 high-confidence novel loci having at least 10 uniquely mapped reads in at least six samples and more than 10X overall sequencing depth. After this refinement, 448 novel isoforms and 778 novel loci that had both high-novelty and high-confidence were identified. There was no association between high-novelty and high-confidence (Figure 9.2).

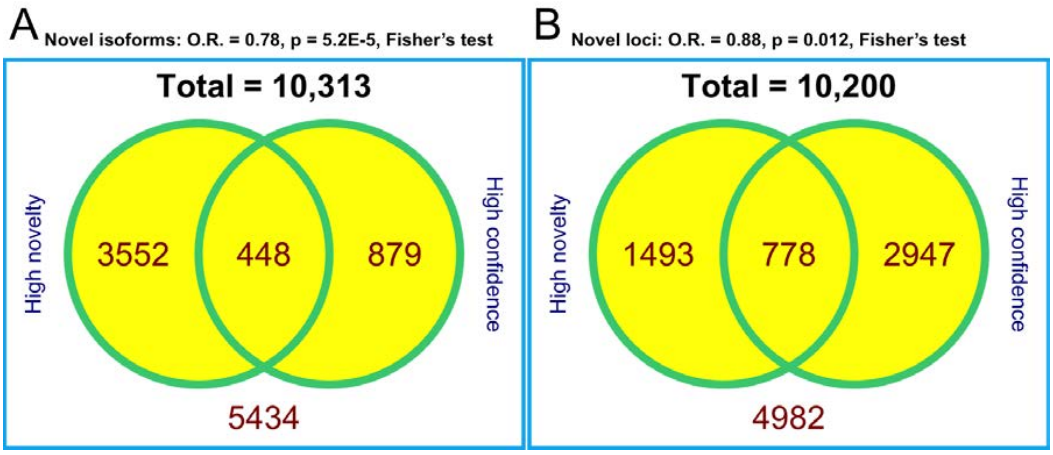


Figure 9.2 – High novelty and high confidence novel isoforms and loci. A. Novel isoforms including at least one unknown exon-exon junction or mapped by ≥10 unique reads in at least six libraries were considering as having high-novelty or high-confidence, respectively. **B.** Novel loci not overlapping any known transcribed region or mapped by ≥10 unique reads in at least six libraries were considering as having high-novelty or high-confidence, respectively. Odds ratios and p values were the result of Fisher Exact test performed on the overlap.

We manually examined a subset of the 778 novel loci for protein coding potential and the possibility that they are orthologs of coding genes in other species using BLAST. Most did not exhibit similarity to any annotated genes and the majority had limited protein coding potential. We validated transcription from 27 novel loci using RT-PCR.

9.4.2 Class-specific transcripts in SLE

The major goal of this study was to identify a unique signature of the monocyte transcriptome in SLE as a strategy to improve our understanding of the pathogenic mechanisms. A large number of known protein coding genes expressed in normal monocytes were found to be silenced in SLE (Figure 9.3A). These genes were highly enriched with the ones related to embryo development (p=6.0E-60), suggesting that SLE monocytes are more differentiated. Antisense transcripts were also more likely to be silenced in SLE (Figure 9.3B). On the other hand, the numbers of novel loci and novel isoforms transcribed only in patients were 3.95 and 1.49 times higher, respectively, than the numbers of control-specific ones (Figure 9.3C and 9.3D).

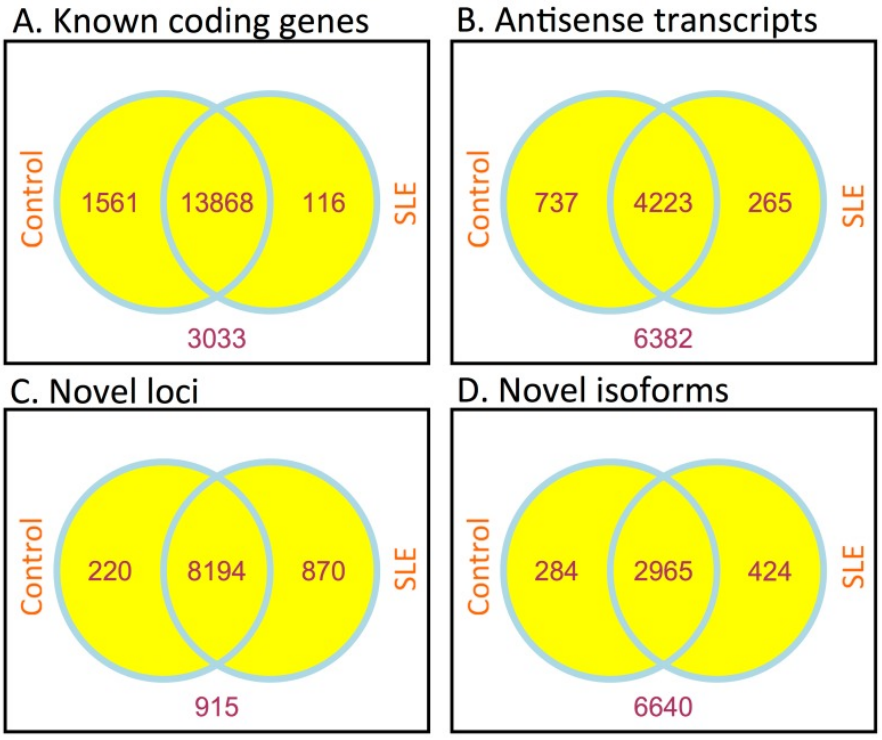


Figure 9.3 – Comparison of SLE and control transcriptomes. The Venn diagrams demonstrate the overlap of actively transcribed **A.** Coding genes; **B.** Antisense transcripts; **C.** Novel loci, and **D.** Novel isoforms in the two sample groups.

We evaluated the differential expression between the two groups of samples in six classes of genes/transcripts: known RefSeq protein coding genes, novel loci identified by Cufflinks, lncRNAs, small RNAs, repetitive elements, and the antisense transcripts of coding genes. These genes/transcripts were filtered by a statistical analysis so all the remaining 33,760 were mapped by at least three sequencing reads in at least three samples. We applied the negative binomial test implemented by the edgeR package of

Bioconductor (911) and identified 1,754 differentially expressed genes/transcripts with p values less than 0.01 and a false discovery rate approximately equal to 0.2. Classes of differentially expressed genes are shown in Table 9.2.

Class	Total Tested	Higher in SLE	Lower in SLE
Known coding gene	14,501	324	329
Novel loci	8,732	659	56
lncRNA	3,482	29	60
Small RNA	379	2	1
Repetitive element	847	11	61
Antisense transcript	5,819	49	171

Table 9.2 – Number of differentially expressed genes/transcripts in each class.

The patient-control difference of total transcription varied dramatically between RNA classes (Figure 9.4A). Total transcription of both sense and antisense transcripts of known coding genes was reduced by approximately 15% in SLE (p=5.6E-61 and 7.4E-105 respectively).

Total transcription of novel loci transcription was strikingly increased by over 45% in SLE (p<1.0E-300), as SLE patients produced many previously undiscovered transcripts that had low or no transcription in healthy monocytes.

Expression of small RNAs was downregulated by about 5%, while expression of 91 pri-miRNAs was dramatically increased by 38% (p=0.01). The three classes of small nucleolar RNAs were all downregulated in SLE patients (Figure 9.4B). The overall up-regulation of pri-miRNAs and down-regulation of coding genes jointly suggested a modified miRNA regulatory system in SLE.

Subclasses of repetitive elements also had different directions of change in SLE (Figure 9.4C). All endogenous retroviral (ERV) subclasses were consistently downregulated (p=4.7E-4 to 3.8E-29). Other retrotransposons such as LTR retrotransposons, SINE, and LINE (L1) elements were variably decreased in SLE patients while expression of the SINE1/7SL element was increased.

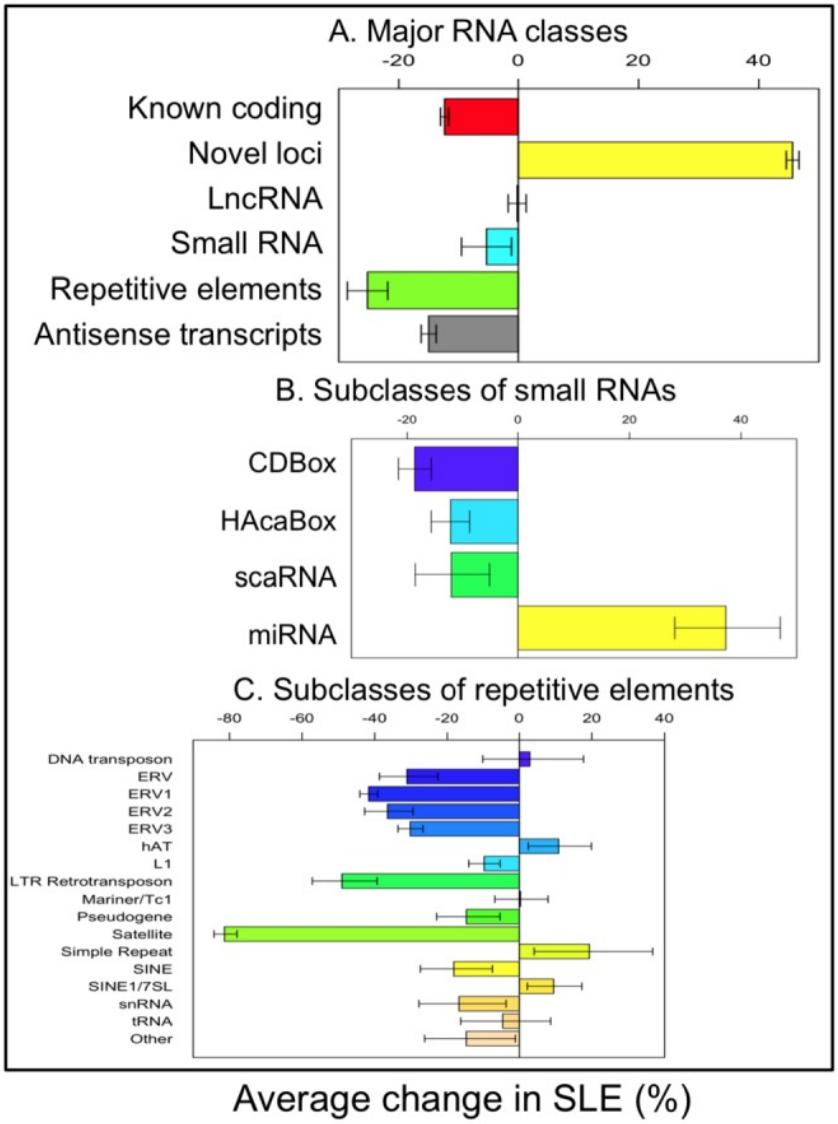


Figure 9.4 – Differential expression of RNA classes. A. The average transcriptional changes of five RNA classes in SLE. **B.** The average transcriptional change of four sub-classes of small RNAs in SLE. **C.** The average transcriptional change of 17 subclasses of repetitive elements in SLE. Error bars indicate standard deviation.

9.4.3 Protein-coding genes

Functional analysis of the protein coding genes identified a large number of pre-defined gene sets whose members were enriched within differentially expressed genes. Most noticeably, differentially expressed genes upregulated in SLE were enriched with genes related to immune response and cytokine activity while the downregulated differentially expressed genes were enriched with genes related to cell adhesion and motion.

	Gene Set	Count	Enrichment	P Value	Representative Genes
Higher in SLE	Cytokine activity (GO:005125)	19	5.67	6.3E-09	*CXCL1, CXCL3, CXCL5, CCL4, CCL20, CCL22*
	Immune response (GO:0006955)	35	2.84	6.5E-08	*C3, TNFSF14, TNFRSF4, NOTCH1*
	Regulation of cell proliferation (GO:0042127)	35	2.49	1.4E-06	*E2F7, CSF1, CSF2, VEGFA, PLAU*
	Genetic association with systemic lupus erythematosus	12	3.79	2.2E-04	*CXCR1, CXCR2, C3, CFB, IL1RN, IL8*
	IFN-induced 56K protein (PIRSF005680)	3	31.25	3.5E-03	*IFIT1, IFIT2, IFIT3*
Lower in SLE	Granules (GO:0031091)	12	12.17	2.8E-09	*SELP, F13A1, GP1BA, PF4, ITGB3, ITGA2B*
	Cell adhesion (GO:0007155)	28	2.42	3.4E-05	*CDHR1, CDHR4, PCDH8, MUC4, MUC5B, MUC16*
	Regulation of cell motion (GO:0051270)	10	3.13	4.7E-03	*BBS1, MAP3K1, VCL*

Table 9.2 – Functional categorization of differentially expressed genes by DAVID.

According to Genetic Association Database (GAD), 11 upregulated differentially expressed genes were associated with SLE in previous studies (Table 9.3).

Gene	SLE/Control Expression	P Value
CFB	562%	2.7E-07
CYP1A1	267%	9.4E-03
HSPA2	1068%	2.0E-05
IL1RN	180%	8.7E-04
IL8	123%	1.9E-03
CXCR1/IL8RA	558%	2.5E-08
CXCR2/IL8RB	289%	1.6E-03
SPP1	310%	6.4E-03
SERPINE1	218%	4.6E-03
C3	206%	2.0E-03
SOD2	139%	3.8E-03

Table 9.3 – Cross-referencing of genes identified in genetic association studies and significantly upregulated in this study.

In addition, the combined gene set of upregulated and downregulated differentially expressed genes was enriched with potential targets of transcription factors highly relevant to SLE, including AP1 (p=3.4E-09), E47 (p=1.1E-8), RFX1 (p=5.8E-7), IRF1

(p=1.4E-3), and IRF2 (p=1.3E-3). We selected six differentially expressed genes to be validated in new samples using qRT-PCR. Five of the genes were significantly different between controls and patients (p=0.049 to 0.0001) and all six were changed in the same direction in both patient cohorts (Figure 9.5).

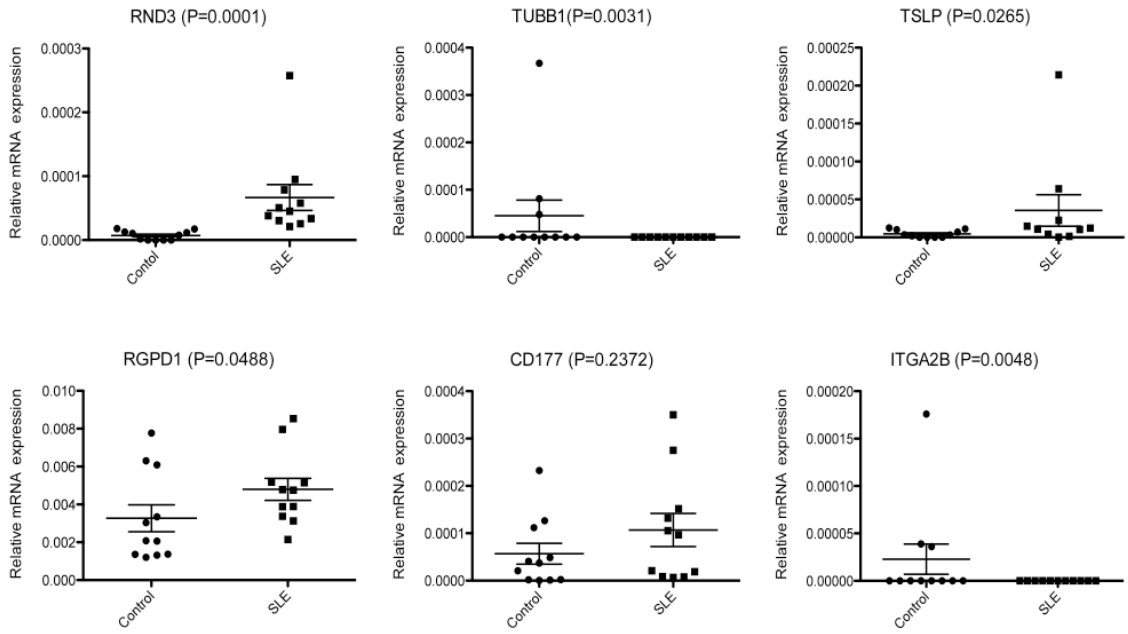
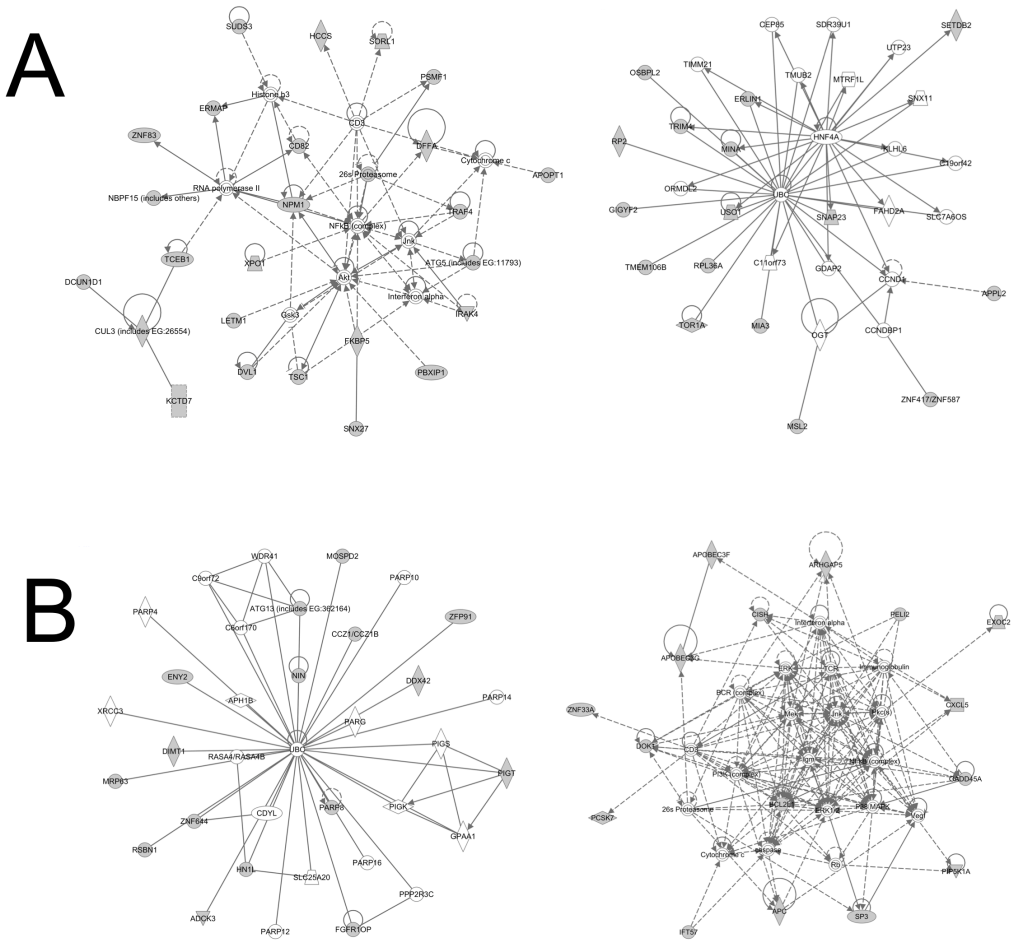


Figure 9.5 - Validation of differential gene expression by RT-PCR. The differential expression of six coding genes in SLE was validated by qRT-PCR. The samples consisted of 11 controls (including 3 internal validation samples from which the RNA-seq libraries were made) and 11 new SLE patients. Five of the genes were validated as having significant change in SLE. The sixth gene, CD177, had the same direction of change in SLE samples but the change did not reach statistical significance. The cross bars indicate mean and standard error according to the Mann-Whitney test.

9.4.4 Polyadenylation

Polyadenylation, along with mRNA cleavage and termination, are regulated in a gene-specific manner and these steps appear to be particularly important for the regulation of genes involved in inflammation (912–914). Tissue-specific and developmentally-regulated polyadenylation have been described with differentiation favoring longer 3' UTRs (915–917). We examined the structure of the coding genes by defining the 3' untranslated region for each gene. Sixty-seven genes had longer 3'UTRs in patients compared to controls and 54 genes had shorter 3'UTRs in patients (defined by a ratio of >2.0 and a p<0.01). The longer 3'UTR gene set was characterized by pathways centered on NFκB, AKT, UBC and HNF4A. The shorter 3'UTR gene set was characterized

by pathways centered on UBC, NFκB, and ERK (Figure 9.6). These gene sets are notable and may give insight into the SLE process (916,918).



Long UTR Pathway Analysis		
Category	**P value**	**# molecules**
Cancer	1.5×10^{-4}	29
Antimicrobial Response	3.9×10^{-5}	3
Cell Morphology	2.2×10^{-5}	13
Cellular Assembly	2.3×10^{-4}	9

Short UTR Pathway Analysis		
Category	**P value**	**# molecules**
Cancer	3.2×10^{-3}	9
Cell Cycle	6.4×10^{-4}	9
Cellular Assembly	2.2×10^{-3}	13

Figure 9.6 – 3' UTR length networks. A. The genes with longer 3'UTRs in SLE patients were networked using Ingenuity. The two most dominant networks are shown. NFκB, AKT, and UBC are the dominant nodes. **B.** The genes with shorter 3'UTRs in SLE patients were networked using Ingenuity. The two most dominant networks are shown. MAP kinases and UBC were the dominant nodes. Data output from Ingenuity is shown in the Tables below.

9.4.5 Antisense transcription

We analyzed antisense transcription because of the potential for sense-antisense duplex RNA to drive type I IFN expression and because antisense transcripts can regulate transcription in *cis* (919). The gene-level read counts of sense and antisense transcripts were positively, but weakly, correlated (r= 0.19; p<0.01). Overall, examining patients and controls together, the total number of reads mapped to the antisense transcripts was about 1/30 of the total number of reads mapped to the sense transcripts. However, a small set of genes demonstrated much higher antisense transcription than sense transcription, such as *USP5*, a ubiquitin peptidase, and *CMTM5*, a chemokine-like factor gene.

In SLE, some coding genes had significantly changed antisense transcription in the opposite direction of their sense counterparts, most noticeably *IVNS1ABP*, *RACGAP1* and *THBS1*. All three genes had significantly upregulated sense transcripts and significantly downregulated antisense transcripts in SLE. The reversed change of sense and antisense transcripts of these genes suggested that the two transcripts are distinctly regulated.

9.4.6 LncRNA expression

LncRNAs can regulate nearby coding genes as *cis*-regulatory elements. We summarized the correlation of transcription between pairs of lncRNAs and coding genes (Figure 9.7).

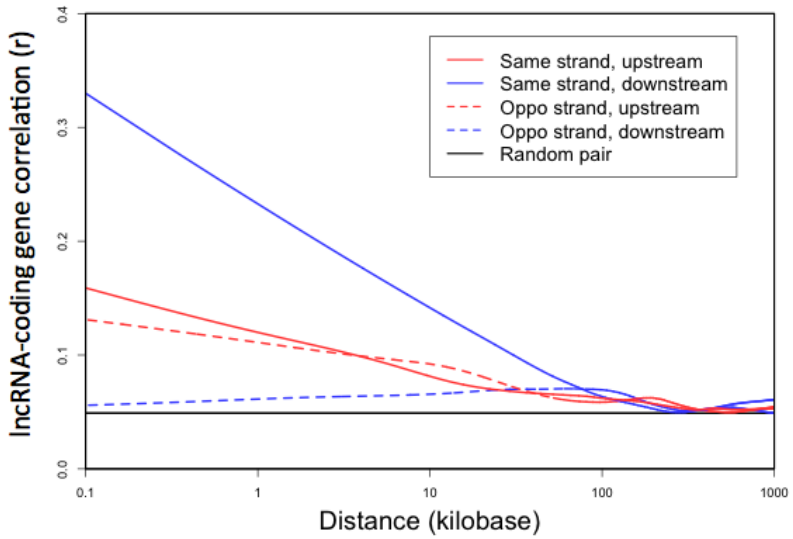


Figure 9.7 - LncRNA association with adjacent transcription. The co-regulation of lncRNAs and their nearby coding genes was dependent on their distance and relative location. The horizontal black line indicates the average correlation of random pairs of lncRNAs and coding genes.

When the lncRNAs were located within 100kb upstream of coding genes, the pairs had a positive correlation on average, regardless of whether the lncRNAs were on the same strand as the coding genes or not. The correlation increased as the distance between lncRNAs and coding genes became closer. On the other hand, when the lncRNAs were located downstream of coding genes, there was no association between the two classes of transcripts if they were on the opposite strands, and there was a much stronger association if they were on the same strand. The latter might suggest the misclassification of extended 3' UTR transcription of coding genes as lncRNAs.

Although lncRNAs were less likely to change in SLE compared to other RNA classes, the locations of some significantly changed lncRNAs suggested their involvement in SLE. For example, both *HIVEP2* itself and a lncRNA about 800 to 1,500 bases upstream of its transcription start site were significantly upregulated in SLE, although their RNA abundance was over 50 times different. There were also two significantly upregulated lncRNAs located within 50kb of each other on chromosome 6 and surrounded by a group of signficantly dysregulated coding genes including *TAGAP*, *FNDC1*, *SOD2*, *WTAP*, and *ACAT2*.

9.4.7 Pri-miRNA expression

Our RNA purification method did not retain miRNAs, however, pri-miRNAs were easily detected and the combination of increased pri-miRNAs and decreased targets suggested an impact of the miRNAs (Figure 9.8).

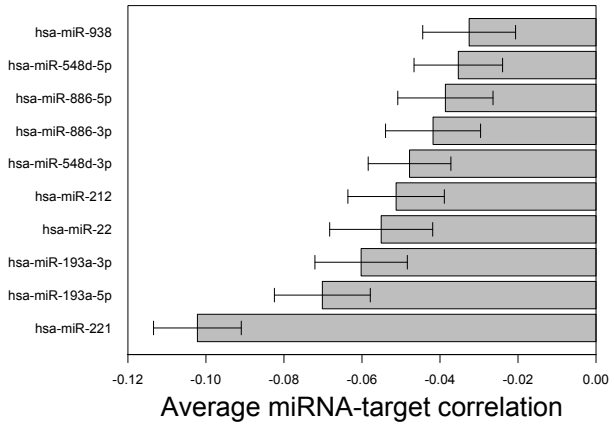


Figure 9.8 – MiRNA – target correlation. Ten pri-miRNAs and their predicted targets had a negative correlation in monocytes. The correlation was calculated using normalized read counts of pri-miRNAs and target coding genes. The correlation coefficients of each miRNA-target pair were obtained from the control and SLE groups separately and then combined using Fisher's transformation. Each bar represents the average and standard error of correlation coefficients of a target list. Target lists were downloaded from miRBase.

Two pri-miRNAs were significantly increased in SLE samples with concomitantly diminished target mRNA levels. MiR-193a regulates k-ras and cell survival and miR-212 regulates apoptosis, two processes known to be aberrant in SLE monocytes (920,921). We validated mature miR-212-3p differential expression in SLE using qRT-PCR on new samples from six controls and 13 SLE patients (Figure 9.9). An exogenous control was used because we could not identify an miRNA in monocytes that was unaffected by cytokine treatment. The expression of miR-212-5p was too low to be detected in this ligation-based detection strategy.

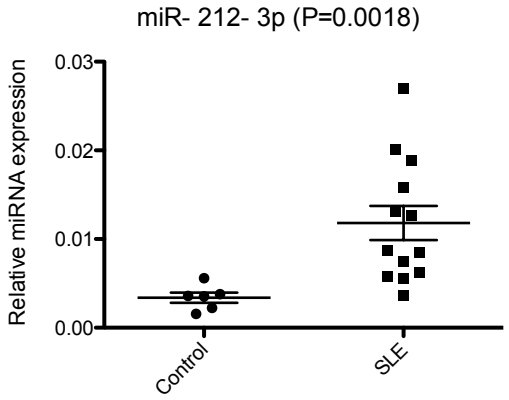


Figure 9.9 – miRNA validation. The increased expression of miR-212-3p in SLE was validated by qRT-PCR with cel-miR-238 as a control. Six new controls and 13 new SLE samples were used.

The predicted targets of miR-193a-3p, miR-193a-5p and miR-212 were downregulated by 14.0% to 16.7% in SLE, which was more than the average down-regulation of all other genes (Figure 9.10).

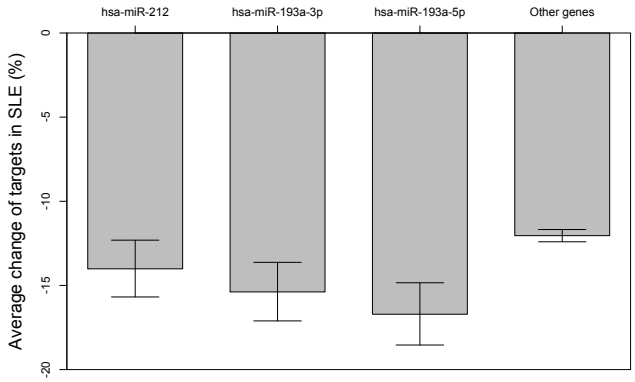


Figure 9.10 – High expression miRNA target transcript expression. Three miRNAs whose pri-miRNAs had higher expression in SLE were examined for an effect on potential target transcripts. For these three miRNAs target transcript expression was significantly downregulated on average. While coding genes were generally downregulated in SLE, the target genes tended to be downregulated even more. Target lists were downloaded from miRBase database.

9.4.8 Repetitive element expression

There were substantially more repetitive elements downregulated in SLE than upregulated ones (Figure 9.4). A closer look at the downregulated repetitive elements showed that they were predominantly ERV classes and were closely correlated with each other, suggesting that they were co-regulated via an upstream mechanism. IFN is known to repress retroviral activation and over-expression of IFN in SLE has been documented in several studies (280,282,922–924). Coding genes having the highest positive correlation with the downregulated repetitive elements were potential targets of transcription factors AP1 (p = 3.0E-8), E47 (p=2.2E-9), RFX1 (p=3.7E-8), IRF1 (p=3.5E-2), and IRF2 (p=4.3E-3), also supporting an effect of IFNs.

9.4.9 Isoform analysis

The proportions of the total reads assigned to each isoform for each gene were compared between the control and SLE samples to evaluate differential isoform transcription. Therefore, this analysis only considered the change of relative isoform abundance while ignoring the gene-level differential expression. This comparison identified 54 genes having one or more isoforms where the relative abundance was significantly changed ($p<0.05$) by at least 5%, such as *CCR2, HIVEP1, HIVEP2, IL1B, IL1R2* and *TLR2*.

SNAPC3 encodes a subunit of a protein complex that activates snRNA. It was previously known as having a single isoform while our Cufflinks assembly identified a novel splicing site within its 3'UTR (Figure 9.11). The relative abundance of these two alternative transcripts was changed by 12% (p=0.003).

We observed that, on average, isoforms where the relative abundance was increased in SLE had significantly more exons than those with decreased relative abundance (Figure 9.12). This result indicates higher RNA splicing activity and/or more complex RNA processing in SLE monocytes.

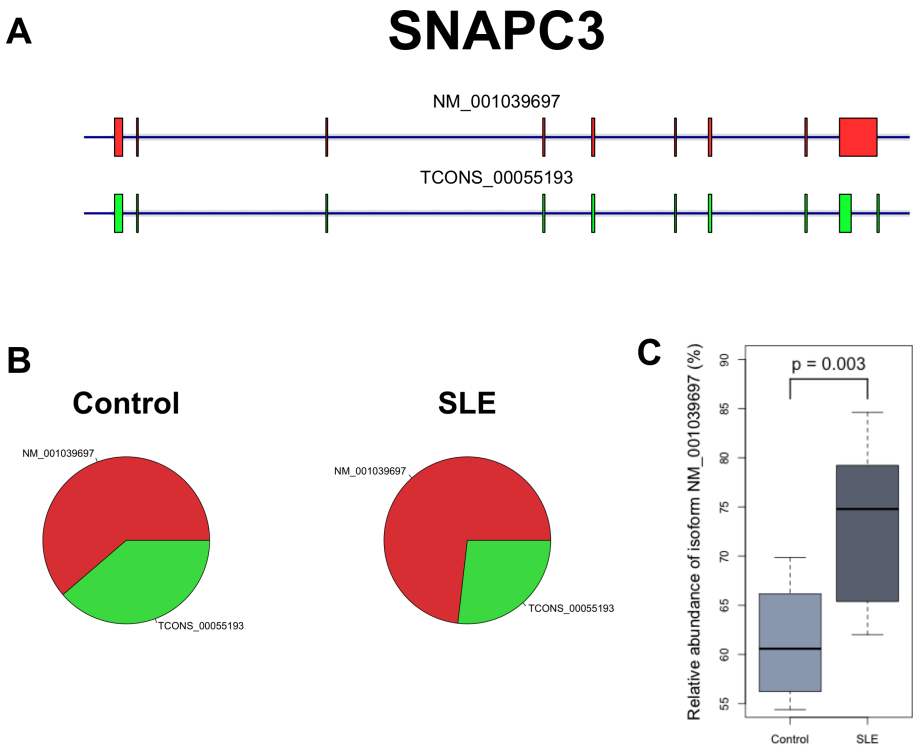


Figure 9.11 – Isoform distribution for *SNAPC3*. **A.** Cufflinks assembly based on our RNA-seq data identified a novel splicing site within the 3' UTR of *SNAPC3*. **B.** The relative abundance of the two isoforms was changed in SLE**. C.** The difference in relative abundance was statistically significant.

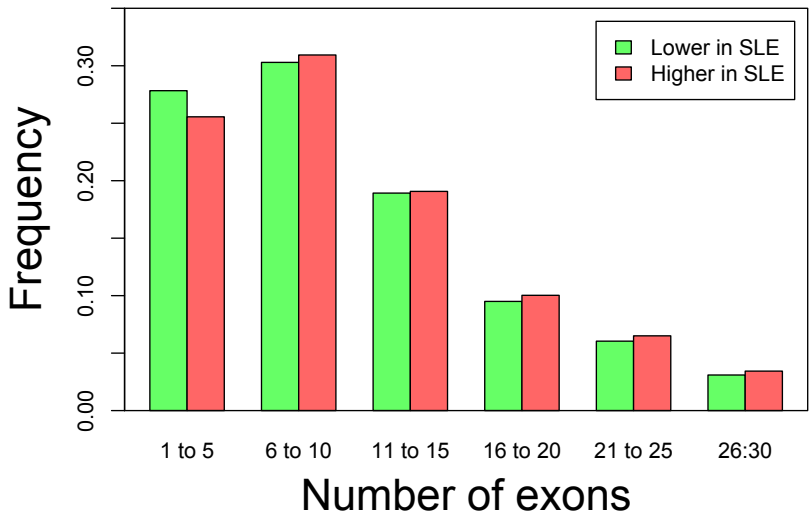


Figure 9.12 – Isoforms with increased expression in SLE are more complex. The isoforms where the relative abundance was increased in SLE had a higher number of exons than those where the relative abundance was decreased ($p = 0.007$). On average, the SLE-favored isoforms had about 0.5 exons more than the control-favored isoforms.

We selected two biologically relevant genes to validate the expression of their novel isoforms in monocytes. Primers were designed to selectively bridge the novel exon-exon junction. *IL1R1* and *IRF8* were both found to include a large intragenic exon by RNA-seq. These exons have no protein coding potential. For IL1R1, the novel exon is an extra 5' untranslated region. In both cases, PCR demonstrated incorporation of the novel exon (Figure 9.13).

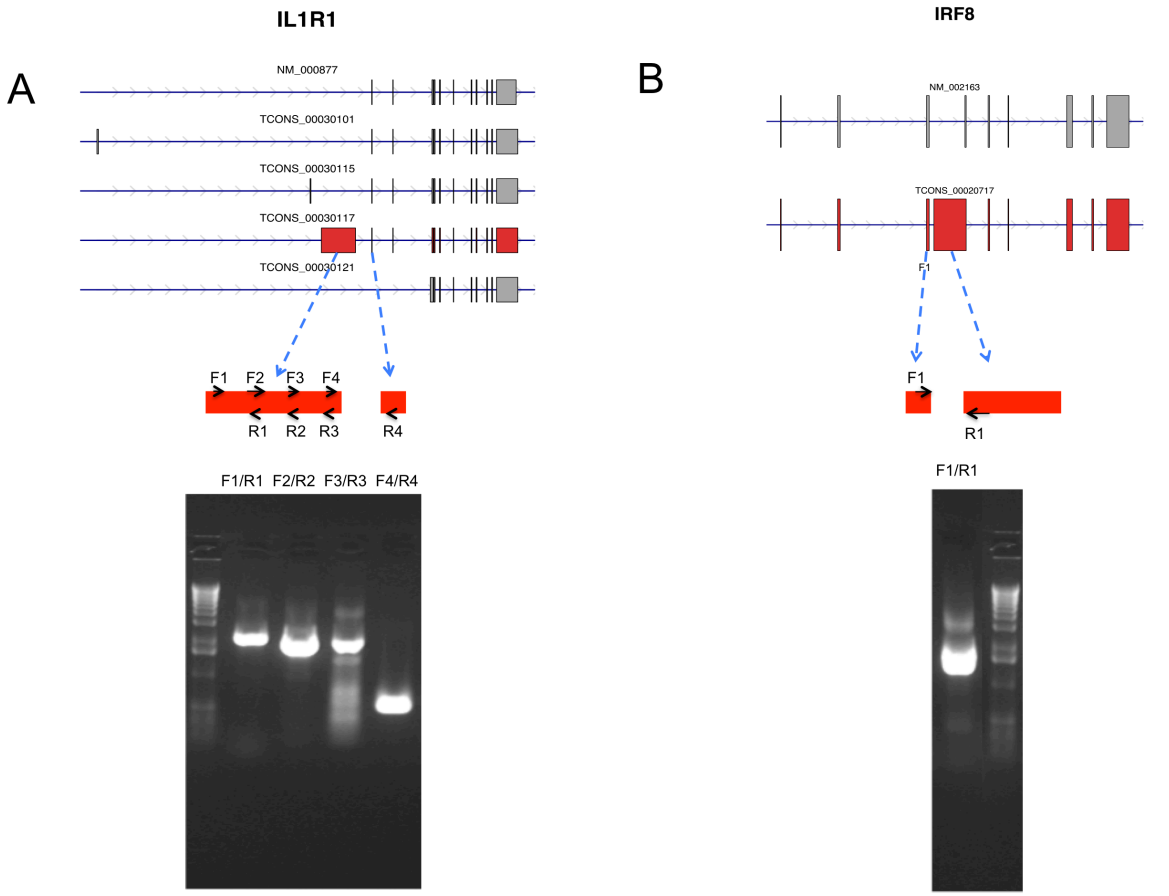


Figure 9.13 – Novel isoform validation. A. Tophat-Cufflinks identified multiple novel isoforms of IL1R1, each with a new exon-exon junction in the 5' UTR. One of the isoforms (in red) was validated by qRT-PCR using a pair of primers across two exons (F4/R4). B. IRF8 was known to have a single isoform. Tophat-Cufflinks identified a novel isoform, which was validated by qRT-PCR. These gels are representative of three experiments, with comparable results.

9.4.10 Novel loci transcription

As shown in Table 9.2, 92% differentially expressed novel loci had higher transcription in SLE. Many of the novel loci formed clusters located close to each other. For example, three of the four novel loci located within a 5kb region on chromosome 18 were among the most significantly upregulated transcripts. Another example was a cluster of 26 novel loci located within a 34kb region on chromosome 8, most of which

were significantly upregulated in SLE and none of which were downregulated. We selected 22 novel transcripts upregulated in SLE to be validated in new samples using qRT-PCR. Ten of the transcripts were significantly upregulated (p=0.034 to 0.0006) in SLE (Figure 9.14), and only two were not upregulated at all. The other loci were upregulated without reaching statistical significance.

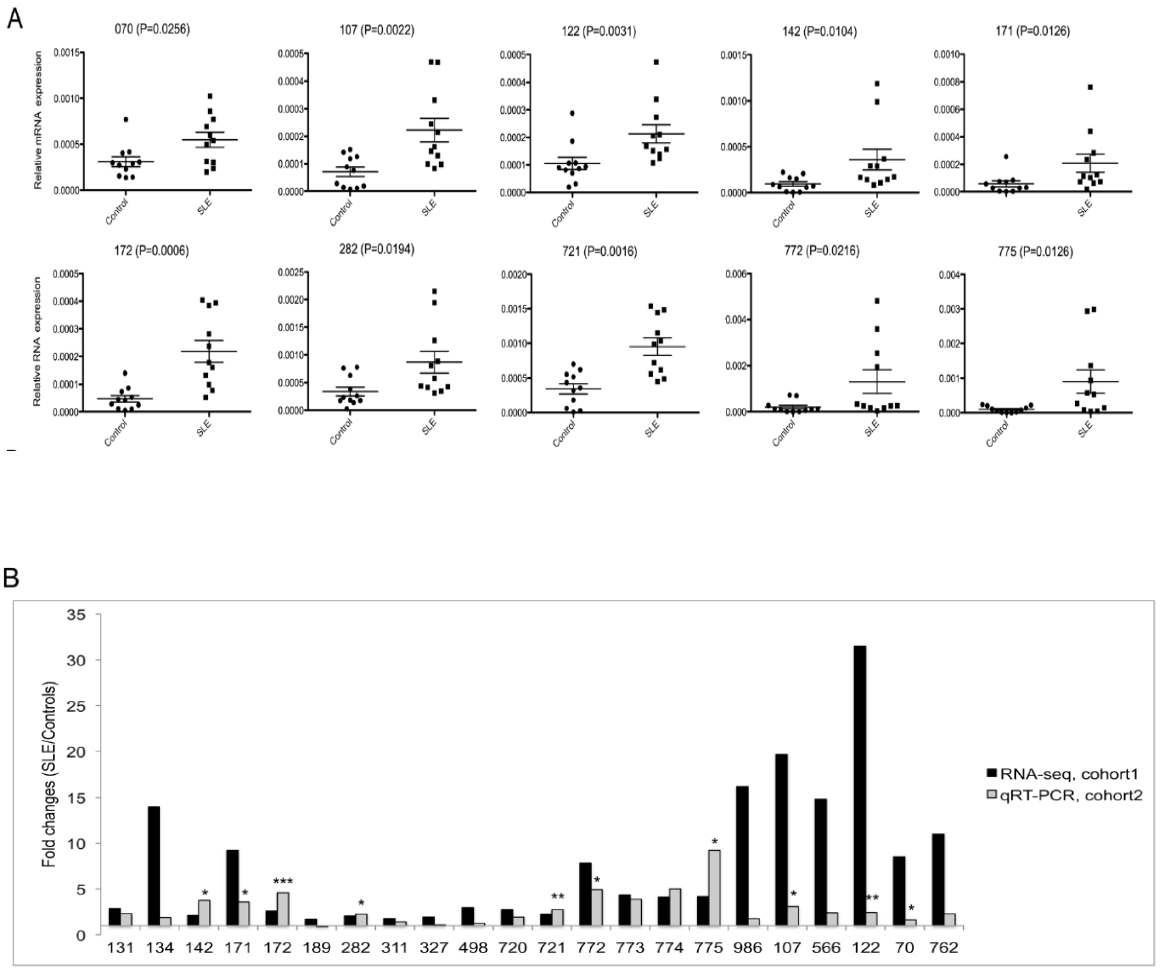


Figure 9.14 – PCR validation in new samples. A. Ten novel loci (70, 107, 122, 142, 171, 172, 282. 721, 772, and 775) were amplified using 11 controls (8 new controls and 3 controls used for the RNA-seq libraries) and 11 new SLE patients. Transcript levels were normalized to β-actin. In each case, the differential expression between SLE and controls was statistically significant with p<0.05, according to the Mann-Whitney test. The cross bars indicate mean and standard error. **B.** There was a good agreement of expression fold changes in SLE between the RNA-seq and qRT-PCR experiments with p<0.05 (*), p<0.01 (**) or p<0.001(***). All but two of 22 tested novel loci were upregulated in both SLE patient cohorts.

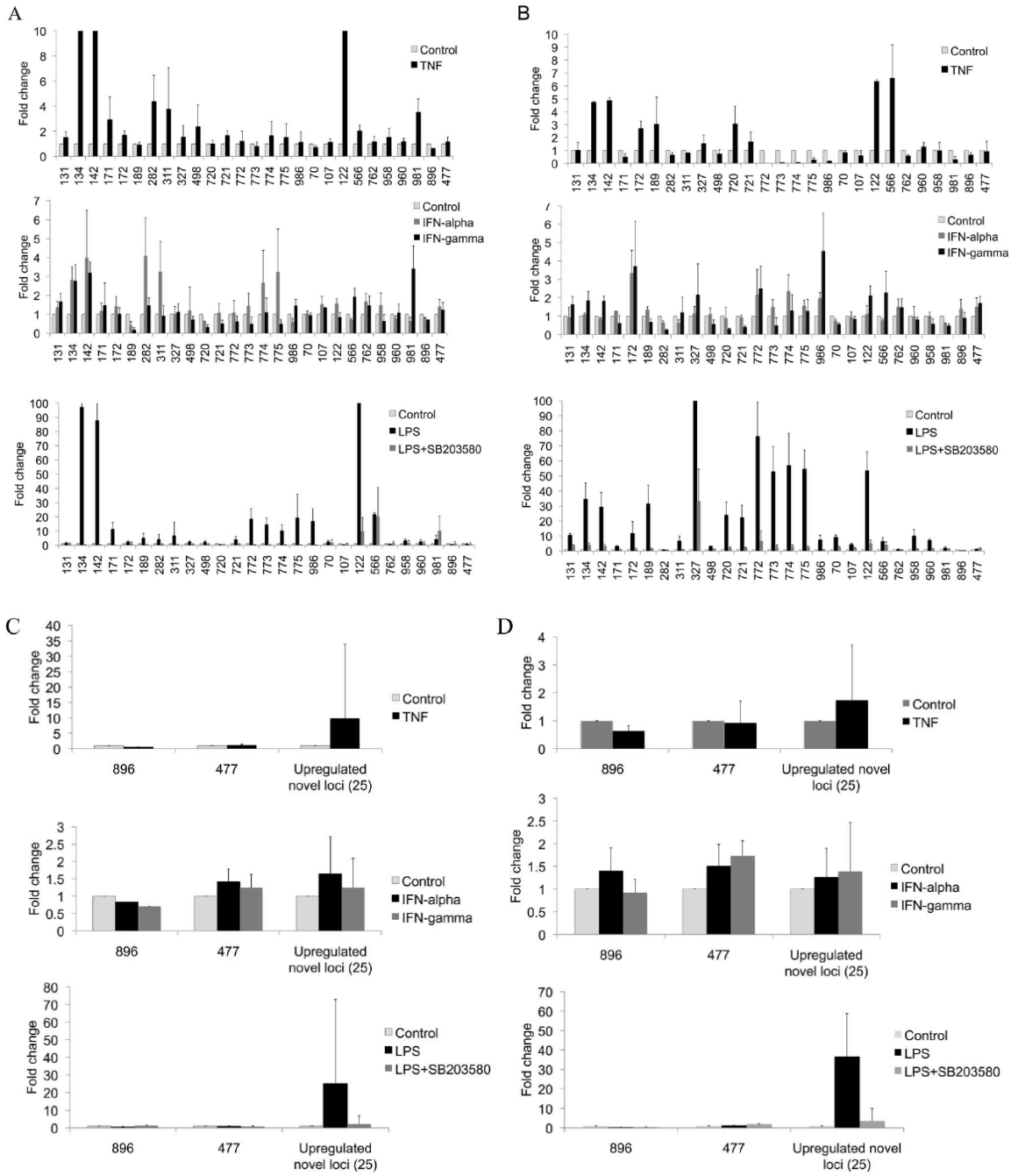


Figure 9.15 – LPS stimulation of monocytes. A. MonoMac 6 cells were stimulated for 16 hours as indicated and qRT-PCR was performed for the novel loci. Only LPS (1 μg/ml) stimulation consistently upregulated these loci. TNF (10 ng/ml) led to upregulation of 16/25 (fold change>1.5), IFNα (100 U/ml) led to upregulation of 9/25 (fold change >1.5), IFNγ (10 ng/ml) led to upregulation of 5/25 (fold change>1.5), and LPS led to upregulation of 22/25 (fold change >1.5). Three experiments with duplicates or triplicates were averaged. The error bars indicate standard deviation. **B.** Human primary monocytes were stimulated as above and the abundance of the novel transcripts quantitated by qRT-PCR. TNF led to upregulation of 9/25 (fold change >1.5), IFNα led to upregulation of 7/25 (fold change >1.5), IFNγ led to upregulation of 9/25 (fold change >1.5), and LPS led to upregulation of 23/25 (fold change>1.5). Pre-treatment of cells with the p38 inhibitor, SB203580 led to markedly diminished induction of expression by LPS. Three experiments with duplicates or triplicates were averaged. The error bars indicate standard deviation. **C.** The average fold change for the aggregated novel upregulated loci in SLE were calculated for MonoMac 6 cells. **D.** Human primary monocytes from healthy donors stimulated by LPS, TNF, IFNα,or IFNγ. Locus 896 was downregulated and locus 477 was not changed in the original SLE samples and were included as controls. The error bars indicate standard deviation in C and D.

The fact that these loci were preferentially or exclusively transcribed in SLE monocytes indicates they are very cell-specific and why they were not previously detected in other cell types and normal monocytes. To examine potential mechanisms driving expression, we initially treated Monomac 6 cells with TNF, IFNα2, IFNγ, or LPS. Time course experiments identified the optimal stimulation time. Only LPS replicated the pattern of expression of novel loci seen in the SLE samples (Figure 9.15). These findings were further validated using published datasets and examining concordance. In this analysis, genes over-expressed in SLE were equally likely to be represented in the IFNα2 - and LPS-induced gene sets (Figure 9.16).

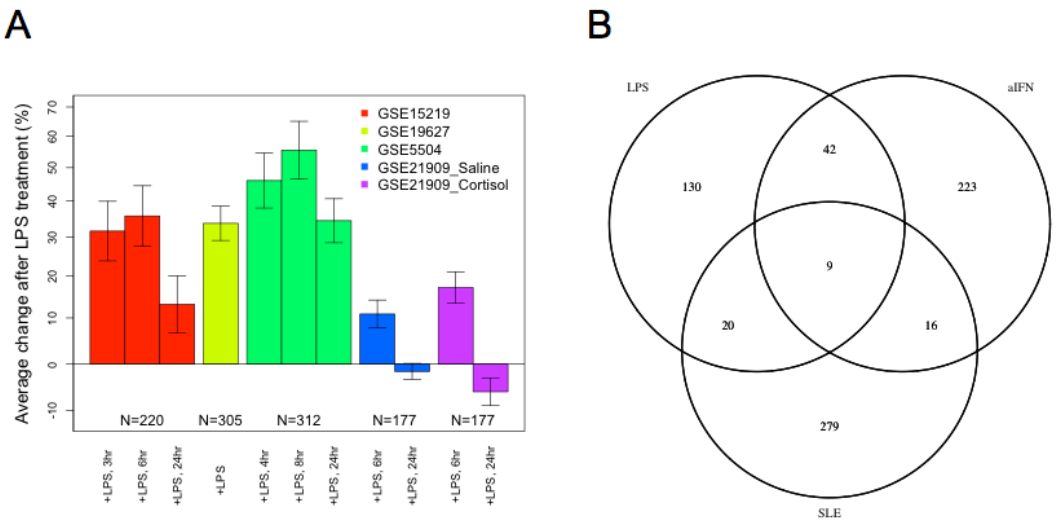


Figure 9.16 – Concordance of LPS, IFN and SLE gene expression. Five LPS data sets were created from four GEO monocyte data series. The coding genes shown to be upregulated in SLE were analyzed for upregulation after LPS stimulation and IFNα treatment. **A.** All LPS data sets demonstrated that LPS treatment also increased expression of the genes shown to be upregulated in SLE. N = the number of unique genes included in the analysis. **B.** The degree of overlap between SLE-induced genes and IFNα-induced genes was comparable to the degree of overlap between SLE-induced genes and LPS-induced genes.

We then confirmed the effect using monocytes from healthy adult donors (Figure 9.15B).

To understand potential pathways regulating the LPS effect, we utilized p38 (SB203580), ERK (U0126) and JNK (SP600125) inhibitors. The major effect of LPS induction of the novel transcripts was p38 mediated (Figures 9.15C and 9.15D and Figures 9.17).

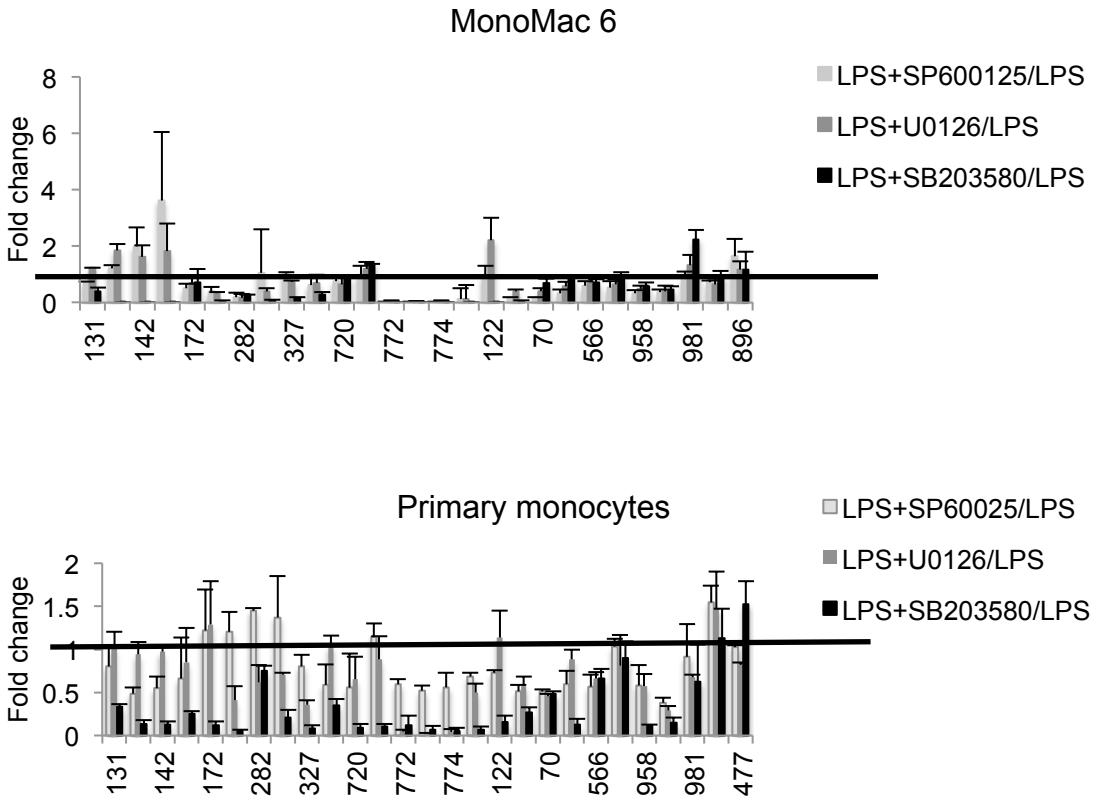


Figure 9.17 – The effect of different MAP kinase inhibitors on LPS-induced novel loci expression. MonoMac6 cells (above) and primary monocytes (below) were treated with p38 (SB203580), ERK (U0126) and JNK (SP600125) inhibitors for 30 minutes and then stimulated with LPS. The y-axis represents the ratio of the LPS + inhibitor-treated cells over the LPS alone treated cells, with the horizontal line indicating equivalence. The p38 inhibitor led to most diminished expression of the novel loci. At the right of the graph, loci 477 and 896 were included as controls, which were novel loci that were not altered in SLE. N = 3.

9.4.11 Circulating endotoxin

Although endotoxin has been previously implicated in murine lupus models and LPS is known to activate IFN pathways via TLR4, there has been no direct measurement of endotoxin in SLE patients (408,925–930). We compared, therefore, serum levels from 99 female SLE patients and those from 112 female healthy adult blood donors. The SLE patients were 91% female, 46% African-American, 54% Caucasian, mean age 37.1 years, mean SLEDAI of 2.6 and mean physician global estimate of 0.45. The mean prednisone dose was 9 mg/day. The Red Cross blood donors were females who were self-declared as healthy. SLE patients had significantly higher endotoxin levels compared to controls (Figure 9.18). When we examined clinical markers for associations with circulating endotoxin levels, we found no association with age, weight, ESR, C-reactive protein, specific organ damage, specific autoantibodies or complete blood count parameters.

Because LPS/endotoxin can induce type I IFN expression, we examined the concordance of coding genes expressed in SLE, after stimulation with LPS and after stimulation with IFNα using our prior results and published array results (550,551). There was substantial overlap, demonstrating that endotoxin can in part mimic the type I IFN signature seen in SLE.

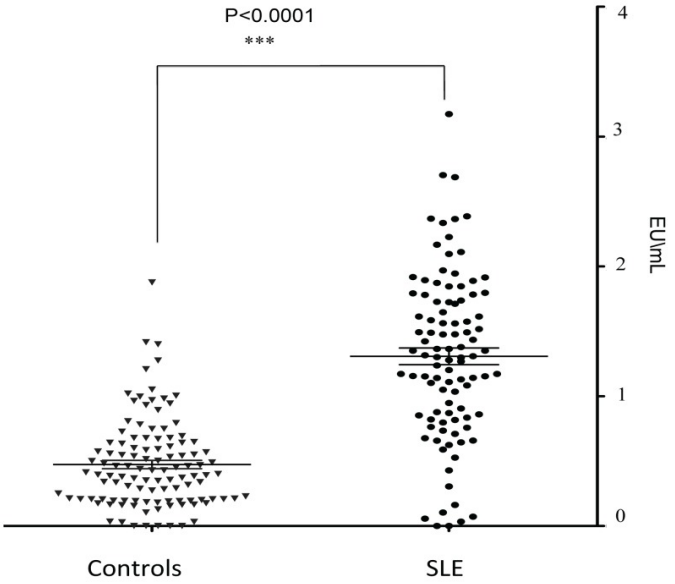


Figure 9.18 – SLE patient circulating endotoxin levels. Circulating endotoxin was quantitated using the Limulus assay. We analyzed 99 female SLE patients and 112 female Red Cross blood donors. SLE patients had significantly more endotoxin on average than controls (p<0.0001). The cross bars indicate the mean and standard error.

9.5 DISCUSSION

With the advent of next-generation sequencing technologies a more comprehensive and accurate transcriptional analysis has become feasible. We report a whole transcriptome analysis of patients with SLE and compare gene expression with that of healthy controls. We detected many instances of SLE-specific alternative splicing, alternative polyadenylation, and novel loci transcription. Splicing and polyadenylation in SLE both favored longer, more complex transcripts.

One of our goals was to identify the transcript abundance of non-coding RNAs, which have been demonstrated in subjects with Aicardi Goutières syndrome, and in murine models, to drive a type I IFN signature (931–933). In this study, we found instead decreased expression of many non-coding RNAs. We hypothesize that repression of

endogenous retroviral sequences may be mediated by the type I IFN that is known to be overexpressed in SLE patients (934).

One class of non-coding RNA for which expression was clearly induced in SLE patients was that of the pri-miRNAs. These small non-coding RNAs are processed to repress translation by augmenting turnover of multiple mRNAs. In our study, two specific pri-miRNAs were significantly upregulated in SLE monocytes compared to healthy controls. In addition we were able to demonstrate that the targets of these two miRNAs exhibited decreased message levels. These observations suggest that the elevated pri-miRNA levels that we identified in SLE monocytes are functionally relevant.

Among known protein-coding genes, there was evidence of global repression, and many genes downregulated in SLE monocytes were related to cell proliferation and cell adhesion. The genes upregulated in SLE monocytes reflected active inflammation. These observations are concordant with what has been seen using arrays and what is known about monocyte behavior in SLE (280,282,549,935–939). Monocytes are known to have a reduced life span and to exhibit characteristics related to type I IFN exposure (280,551).

The finding of SLE-specific isoforms and polyadenylation was particularly intriguing. The finding of disease-specific isoforms is not unique to this study but it has not been reported this extensively outside of tumor-specific transcripts. Some studies have found functional effects of autoantibodies directed at nuclear constituents and altered expression of splicing factors and this study raises the question of the mechanism driving altered processing (940–942). Altered expression of splicing factors could also contribute to the altered pattern observed here (941,942).

This study raises many questions. We examined a cohort of patients with low disease activity to minimize the effect of medications. Whether patients with more severe disease could have a more disturbed transcriptome is not known. This is a realtively small cohort size, and additional studies will be required to replicate these findings. We did not perform extensive validation of the many classes of RNA found to have altered expression, instead capturing a snapshot of the SLE transcriptome in a single cell type and focusing on the breadth of the effect. We validated using new samples from controls and patients for the differentially expressed genes and found similar changes in nearly all, supporting that these effects are consistently seen in the disease.

If similar disruptions are found in additional cell types, it would suggest a systematically altered transcriptome. Additional information is likely to be forthcoming, allowing improved understanding of the regulation of these processes. Monocytes represent a cell type that is uniquely plastic and it may be that effects are magnified in this cell type.

We previously identified IRF1, IRF2, and RFX1 as potential regulators of genes with altered histone H4 acetylation (549–551). These transcription factors, now identified as potential regulators of the SLE transcriptome, could integrate inflammatory and IFN signals. The MAP kinase and NFkB pathways were identified as potential regulating pathways in the polyadenylation pattern specific to the SLE samples. These pathways have long been implicated in SLE (943–948).

To further implicate the MAP kinase pathway, we found that expression of many novel loci was inducible with LPS and that it could be blocked with the P38 inhibitor, SB203580. The roles of the novel loci are not yet known and we acknowledge these fall into a bioinformatic limbo. Nevertheless, endotoxin has been implicated in a variety of diseases and induces type I IFN. Our findings of significant overlap of the genes induced by LPS, SLE and IFNα along with increased endotoxin in peripheral blood supports a role for LPS in the pathologic gene expression pattern identified here.

Limitations of this study include a small sample size focused on mild to moderate disease activity. While the low disease activity enabled us to examine patient samples without the perturbation of high-level immune suppression, it may also have limited our findings. The platform used and the sample cell count may also have limited our findings. The libraries were 50 bp single reads and total RNA was used with post-run ribosomal RNA exclusion. Finally, technical aspects such as RNA quality may have limited our ability to identify disease-specific variation in signal. Nevertheless, in spite of these potential limitations, our analyses were robust and identified many changes specific to the SLE transcriptome.

9.6 CONCLUSIONS

We found a broadly altered transcriptome in SLE. By using a single cell type, we minimized the effects of different cell populations and improved the specificity of our discoveries. The most significant finding of this study was identification of disease-specific novel locus expression, regulated by endotoxin. Circulating endotoxin has been negatively associated with prognosis in a number of diseases and is thought to drive a type of immune exhaustion (949,950). Whether endotoxin could be responsible for other features of the altered transcriptome or could represent a biomarker for disease severity remains to be determined. While additional studies will be required to determine which features contribute to the pathologic processes in this still enigmatic disease, the importance of this studies lies in the identification of multiple features of altered transcription and processing in SLE, a heretofore unappreciated facet of the disease.

This is the first RNA-seq study of SLE and we found not only a transcriptome that exhibits quantitative alterations as defined by the level of gene expression but also qualitative differences with widely altered splicing preference and non-coding RNA transcription. Some novel transcripts expressed at higher abundance in SLE monocytes were inducible by LPS, known to activate type I IFNs. LPS and microbial products have been demonstrated to accelerate renal disease and induce lupus-like processes in mice (927), leading to the hypothesis that circulating endotoxin may be in part responsible for the type I IFN signature in SLE.

Please refer to **Appendix B.2** for the paper ***The SLE Transcriptome Exhibits Evidence of Chronic Endotoxin Exposure and Has Widespread Dysregulation of Non-Coding and Coding RNAs***, which was published in the journal *PLOS one.*

CHAPTER 10

Final Remarks and Future Directions

As a Pediatrician I feel extremely challenging to take care of patients with juvenile-onset SLE. The great heterogeneity of phenotypes and the lack of robust biomarkers to predict the course of the disease are important obstacles. Furthermore, the current treatment strategies, including the long-term use of steroids, are associated with important side effects that have a profound effect in the quality of life of our patients. The burden of SLE is certainly greater in children who have to physically and cognitively grow while facing a chronic disease. Fatigue, depression, chronic pain, as well as multiple clinical visits and, often, the need to be admitted to the hospital, disrupt their youth and make them feel different from their peers. The high rates of school failure and unemployment in this group of patients urge us to do better and to find new ways to improve the care of SLE patients. The main quest is to better understand the mechanisms of perpetuation of the disease, in order to transform it from chronic to curable.

I used the modern tools of molecular biology to try to unveil new clinically relevant pathways in SLE. In collaboration with my colleagues in the lab, I participated in the study of the transcriptome of monocytes and showed that in SLE there is a profound dysregulation of gene expression, not only quantitatively, but also qualitatively. Once again, it was revealed the interesting connection between the microbiome and autoimmunity, since the novel transcripts identified in SLE were replicated when control monocytes were exposed to LPS. Furthermore, we showed that SLE patients have increased levels of circulating endotoxin. This observation propels the study of the gut integrity in this group of patients and its impact on disease activity and chronicity. Furthermore, it opens the door for new studies on the effect of diet and of manipulating the microbiome to control the disease.

During my Ph.D., I mainly focused on two of the most severe manifestations of SLE: lupus nephritis and macrophage activation syndrome. Considering the latter, I was intrigued by hemophagocytes and by their function. Surprisingly, we demonstrated that hemophagocytes from a mouse model of secondary hemophagocytic syndrome have a transcriptome similar to M2 macrophages. Moreover, in a very diverse cohort of patients with hemophagocytic syndromes, we showed that hemophagocytes universally express CD 163, an M2 marker. These data work against the traditional notion that hemophagocytes are inflammatory cells responsible for the cytopenias seen in

macrophage activation syndromes. Hemophagocytes are now presented as possible regulatory cells, which opens a new era for the study of hemophagocytic syndromes.

Regarding lupus nephritis, the miRNA kidney signature of this disease was characterized using a high-throughput methodology. It was found that miR-26a and miR-30b were significantly decreased in the kidneys and in the urine of lupus nephritis patients. *In vitro*, it was shown that these miRNAs control the proliferation of mesangial cells, one of the key steps in lupus glomerulonephritis. Interestingly, another group had shown that these two miRNAs were also decreased in breast cancer cells and that their levels were controlled by HER2. We hypothesized that HER2 also had a role in lupus nephritis and we found a striking overexpression of HER2 in the tubular and glomerular compartments of patients with lupus nephritis. The same pattern was not seen in the kidneys from healthy individuals or in other proliferative glomerulonephritis. We showed that HER2 expression was driven by IFNα and IRF1. Finally, we demonstrated that HER2 was increased in the urine of lupus nephritis patients and that its levels correlated with disease activity and with the levels of other well-known lupus nephritis biomarkers, namely MCP1 and VCAM1.

The results from this research led to original conclusions, which can be translated into the clinic in the near future. HER2 was identified as a new biomarker for lupus nephritis and it can easily be used in the clinical setting, since there are commercially available ELISA kits for HER2 quantification. In addition, PET scans targeting HER2 have been developed to identify metastases of $HER2^{+}$ breast cancer. This methodology is, therefore, available to evaluate the cell proliferation status of lupus nephritis patients. Most importantly, this work also showed that anti-HER2 drugs, already approved for different types of cancer, could also have a role in lupus nephritis treatment, opening the door to a new treatment strategy for this disease.

Finally, on a personal note, I must emphasize that being devoted to research during three years of my life was an extraordinary and truly rewarding experience. I am deeply thankful for this opportunity and I am looking forward to the enormous challenges that will follow.

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PUBLICATIONS

First Author

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1. Genetics and Epigenetics of Systemic Lupus Erythematosus

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Appendix A.1

2. Major Infections in a cohort of 120 patients with Juvenile-onset Systemic Lupus Erythematosus

Authors: Patrícia Costa Reis, Simona Nativ, Josephine Isgro, Cagri Yildirim-Toruner, Amy Starr, Lisa Imundo, Lisa Saiman and Andrew Eichenfield
Journal: *Clinical Immunology*
Date: December 2013
PMID: 24211846
Appendix A.2

3. The Role of miRNAs and Human Epidermal Growth Factor Receptor 2 in Proliferative Lupus Nephritis

Authors: Patrícia Costa Reis, Pierre A. Russo, Zhe Zhang, Lucrezia Colonna, Kelly Maurer, Stefania Gallucci, Steffan W. Schulz, Adnan N. Kiani, Michelle Petri and Kathleen E. Sullivan.
Journal: *Arthritis and Rheumatology*
Date: September 2015
PMID: 26016809
Appendix A.3

4. *Uma Nova Era no Diagnóstico e no Tratamento das Síndromes Hemofagocíticas*

Authors: Patrícia Costa Reis, Sofia Almeida and Edward M. Behrens.
Journal: *Ata Pediátrica Portuguesa*
Date: October 2016
Appendix A.4

5. Alternative Activation of Laser-captured Murine Hemophagocytes

Authors: Scott W. Canna, Patrícia Costa Reis, William E. Bernal, Niansheng Chu, Kathleen E. Sullivan, Michele E. Paessler and Edward M. Behrens.

Patrícia Costa Reis, Scott W. Canna and William E. Bernal contributed equally to this work.

Journal: *Arthritis and Rheumatology*
Date: June 2014
PMID: 24470386
Appendix A.5

Co-author

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6. New insights into the immunopathogenesis of systemic lupus erythematosus

Authors: George Tsokos, Mindy Lo, Patrícia Costa Reis and Kathleen E. Sullivan
Journal: *Nature Reviews Rheumatology*
Date: November 2016
PMID: 27872476
Appendix B.1

7.**The SLE Transcriptome Exhibits Evidence of Chronic Endotoxin Exposure and has Widespread Dysregulation of Non-coding and Coding RNAs**

Authors: Lihua Shi, Zhe Zhang, Angela M. Yu, Wei Wang, Zhi Wei, Ehtisham Akhter, Kelly Maurer, Patrícia Costa Reis, Li Song, Michelle Petri and Kathleen E. Sullivan
Journal: *PLoS ONE*
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Appendix B.2

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APPENDICES

A. PAPERS AS FIRST AUTHOR

Appendix A.1 – Paper ***Genetics and Epigenetics of Systemic Lupus Erythematosus***, published in *Current Rheumatology Reports,* September 2013.

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SYSTEMIC LUPUS ERYTHEMATOSUS (M PETRI, SECTION EDITOR)

Genetics and Epigenetics of Systemic Lupus Erythematosus

Patrícia Costa-Reis · Kathleen E. Sullivan

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Abstract Genetics unquestionably contributes to systemic lupus erythematosus (SLE) predisposition, progression and outcome. Nevertheless, single-gene defects causing lupus-like phenotypes have been infrequently documented. The majority of the identified genetic SLE risk factors are, therefore, common variants, responsible for a small effect on the global risk. Recently, genome wide association studies led to the identification of a growing number of gene variants associated with SLE susceptibility, particular disease phenotypes, and antibody profiles. Further studies addressed the biological effects of these variants. In addition, the role of epigenetics has recently been revealed. These combined efforts contributed to a better understanding of SLE pathogenesis and to the characterization of clinically relevant pathways. In this review, we describe SLE-associated single-gene defects, common variants, and epigenetic changes. We also discuss the limitations of current methods and the challenges that we still have to face in order to incorporate genomic and epigenomic data into clinical practice.

Keywords Lupus · Systemic lupus erythematosus · SLE · Genetics · Epigenetics · Autoimmune diseases

This article is part of the Topical Collection on *Systemic Lupus Erythematosus*

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with a spectrum of clinical manifestations and outcomes. In spite of this variability, epidemiological data indicating a higher concordance ratio between monozygotic twins (24–69 %) compared to dizygotic twins or siblings (2–5 %) have made the role of genetics in SLE indubitable [1]. Nevertheless, single gene defects related to lupus-like phenotypes have infrequently been described and patients with monogenic causes of SLE are thought to comprise only about 1 % of most adult SLE cohorts. The majority of the identified genetic SLE risk factors are, therefore, common variants, with a modest magnitude of risk, which suggests that different mechanisms contribute to the pathogenesis of this disease, including epigenetic factors, which are just starting to be identified.

The proteins encoded by the SLE-associated genes participate in a multiplicity of mechanisms, including: monocyte, neutrophil, B and T-cell function; antigen presentation; type I interferon, toll-like receptor (TLR) and NFκB signaling; apoptosis, and clearance of cellular debris and immune complexes. Some SLE susceptibility variants are also associated with other autoimmune diseases, which may reflect common molecular pathways.

The human leucocyte antigen (HLA) region is the most gene-dense region in the human genome, including 120 functional genes, many of those with a role in immunity [2]. This region was identified as the strongest determinant of SLE predisposition in all the genome-wide association studies (GWAS) performed [3–6]. Furthermore, variants of *HLA-DRB1* were associated with SLE in multiple ethnic backgrounds and an *HLA-DR3* polymorphism (rs2187668) seemed to have an impact on the propensity to produce autoantibodies in SLE [7•].

In this review, we will focus on non-HLA genetic risk factors for lupus. Single-gene defects will be briefly described, followed by a summary of the variants and the broad epigenetic changes that have been associated with SLE.

Single Gene Defects and SLE

Single gene defects have been recognized as causing lupus since the 1970s. Specifically, complete deficiencies of C1q, C1r, C1s, C2, and C4 are strongly associated with SLE. A penetrance higher than 90 % occurs in *C1Q*, with lower penetrance for *C4* (75 %) and *C2* (10–30 %) [8, 9]. The role of complement on immune complexes and apoptotic body clearance is thought to be the underlying mechanism responsible for this association. Although partial deficiencies of C4 and Mannose-Binding Lectin (MBL) have been described as predisposing for SLE [10, 11], large-scale studies did not support this finding, so it seems unlikely that they markedly increase the susceptibility to lupus. They may, however, modify the disease phenotype [12].

Less commonly described are the associations of chronic granulomatous disease (CGD) and the carrier state for X-linked CGD with discoid and systemic lupus [13–16], presumely due to an inability to clear apoptotic cells.

The apoptotic pathway is also affected in autoimmune lymphoproliferative syndrome (ALPS). *FAS* and *FASL* are the genes related to classic ALPS, which have been associated with SLE predisposition [17–19]. Caspase 8 deficiency has similar features and is often categorized as an ALPS disorder, but the immunodeficiency dominates the phenotype. The mechanism of autoimmunity is not fully understood, but may relate to the excess of cytokines, like IL-10 and B-cell activating factor (BAFF), that can break B-cell tolerance.

Finally, approximately 10 % of the patients with prolidase deficiency develop lupus [20]. Cutaneous manifestations are common, but nearly all of the lupus end-organ effects can be seen. Prolidase participates in proline recycling, and its deficiency is thought to lead to apoptosis of cells where collagen synthesis is critical. The true mechanism, however, is not fully understood.

SLE Associated Variants Divided by Their Proposed Mechanisms

Apoptosis and Clearance of Nuclear Debris

In SLE, there is an imbalance of apoptosis and clearance of nuclear debris, which increases the availability of autoantigens, contributing to autoimmunity. Accordingly, several genes related to these mechanisms have been associated with SLE. One example is *ATG5* (autophagy related 5). Several variants of this gene, which encodes for a protein that participates in caspase-dependent apoptosis and autophagy, have been described in European SLE patients [5]. Another example is TREX1 (three prime repair exonuclease), which participates in DNA degradation, granzyme A activated apoptosis and oxidative stress response. *TREX1* null mutations are associated with Aicardi-Goutières syndrome, a disease with lupus-like features, and familial chilblain lupus. Certain *TREX1* variants were found to be related to SLE suceptibility [21] and, in a large case–control study, a *TREX1* haplotype was found to be associated with the risk of neurological manifestations in European SLE patients [22]. In addition, mutations in *ACP5* (acid phosphatase 5, tartrate resistant), which encodes a protein that participates in lysosomal digestion, were shown to cause bone dysplasia, as well as an increase on α-interferon and multiple autoimmune diseases, including SLE [23]. Although polymorphisms in *ACP5* have not been identified in GWAS, its major substrate, osteopontin, has been found in several studies as disease associated [24]. Finally, in a recent study of patients with African ancestry, several novel associations were found between variants of genes associated with the production of reactive oxygen species and SLE [25]. Collectively, these findings demonstrate the critical role of clearing nuclear debris in SLE pathogenesis.

Clearance of Immune Complexes

Genome-wide analysis and candidate gene association studies of diverse human populations showed a consistent linkage to 1q21.1-24, a region that includes the receptors that recognize the constant (Fc) portion of immunologlobulin (Ig) isotypes (FcγRs).

FcγRs can activate (FcγRI, FcγRIIA/C, FcγRIII) or inhibit (FcγRIIB) cellular functions, such as phagocytosis, antibody-dependent cellular cytotoxicity, degranulation, antigen presentation, B-cell activation, cytokine production and immune complex clearance. Numerous single nucleotide polymorphisms (SNP) and copy number variants have been characterized in the FcγR genes. Several of those variants have been associated with an increased risk for SLE. For instance, H131R of *FCGR2A* is a common variant that was shown to have lower affinity for the ligand, leading to a profound decrease on the phagocytosis of IgG2 opsonized particles [26]. The also lower IgG binding *FCGR2A* allele 158 F was associated with an increase risk for SLE in Caucasians [27], but not in an African-American population [28]. Another example is the single amino acid substitution that occurs on the I232T variant of *FCGR2B*, which was also associated with SLE in Asian populations [29, 30], but not in Caucasians [31]. Defective signaling by the risk *FCGR2B* variant increases the inflammatory response of macrophages to immune complexes, reduces the threshold for antigen presentation by dendritic cells and facilitates autoreactive B-cell activation [32], thus contributing for autoimmunity.

FCγR variants are not only associated with disease susceptibility, but also with disease progression and phenotypic features. Variants of *FCGR3A*, for example, were associated with end-stage renal disease in patients with lupus nephritis [33, 34].

Finally, copy number variation is common in regions of the genome coding for immune related genes and it is also associated with SLE predisposition, namely a low copy number variation at the *FCGR3B* locus was associated with SLE and it affected the immune complex uptake by neutrophils [35].

Complement has a dual role in SLE. On the one hand, there is clear evidence that complement activation contributes to the pathogenesis of the glomerular injury that occurs in lupus nephritis. On the other hand, complement participates in the clearance of immune complexes and apoptotic bodies. As previously discussed, complete deficiencies of complement are among the strongest known genetic risk factors for SLE. Moreover, genes associated with the regulation of the alternative complement pathway have also been recently found to contribute to SLE risk, namely genes encoding complement factor H regulator (CFHR) and five-related CFHR-proteins [36].

Toll Like Receptors and α- Interferon Pathway

Type I interferons (α and β interferon) participate in anti-viral immune responses as key regulators of the proliferation, differentiation, survival and activity of the majority of the immune cells [37]. Increased expression of α-interferon and its regulated genes has been described in SLE [38–42] and propelled the development of α-interferon inhibitors for the control of this disease. A number of variants in the receptors that recognize nucleic acids (TLRs), their regulatory molecules (UBE2L3), downstream transcription factors (IRFs, ETS1) and the interferon signaling pathway itself (TLK2) have been described in association with SLE. This large family of variants is a testament of the importance of this pathway in SLE etiopathogenesis.

TLR activation contributes to the production of type I interferons, which may explain the solid evidence connecting TLRs to SLE pathogenesis. One of the possible examples is the association between a functional variant of *TLR7* and SLE in an Asian population [43]. Other robust SLE associations were found with variations in genes coding for the interferon regulatory factors (IRFs): IRF5, IRF7 and IRF8 [44], the transcription factors downstream of TLRs. IRF5 is a transcription factor that induces the expression of multiple pro-inflammatory cytokines, including α-interferon, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-17, IL-23, MCP1 (monocyte chemotactic protein-1), and RANTES (regulated on activation, normal T cell expressed and secreted) [45]. IRF5 is associated with SLE, as well as other autoimmune diseases, including rheumatoid arthritis, Sjogren's syndrome, systemic sclerosis, multiple sclerosis, and inflammatory bowel disease [46]. The IRF5 locus was implicated in SLE through candidate gene analysis [47] and later confirmed by multiple independent case–control cohorts [48–51] and GWAS [4–6, 7•]. Several IRF5 insertion and deletion polymorphisms and SNPs have been described in association with increased or decreased levels of IRF5, α-interferon and, consequently, SLE susceptibility [52, 53]. Interestingly, IRF5 is necessary for the development of lupus-like disease in mice, which demonstrates the importance of this transcription factor in SLE pathogenesis [54]. *IRF7* variants also contribute for SLE predisposition. An *IRF7* SNP (Q412R) is associated with an increase in IRF7 levels and SLE risk in several ancestral populations [55] and additional *IRF7* risk alleles have been associated with anti-double stranded DNA antibodies and anti-Sm antibodies [56, 57]. UBE2L3 (Ubiquitin-conjugating enzyme E2 L3) is known to participate in the degradation of TLRs and genetic variations in *UBE2L3* were also identified as predisposing for SLE and other autoimmune diseases [5, 6, 7•, 58, 59]. ETS1 (v-ets erythroblastosis virus E26 oncogene homolog 1 avian) is a transcription factor that binds the interferon-stimulated response elements, controlling type I interferon-induced transcription. It also participates in the inhibition of Th17 and B-cell differentiation. Evidence of animal models supports the role of ETS1 in SLE, since *Ets1*-deficient mice develop a lupus-like phenotype, characterized by the production of autoantibodies, glomerulonephritis and local activation of complement [60]. In humans, *ETS1* was identified as one of the loci associated with SLE predisposition [6, 61, 62]. Finally, *TYK2* (tyrosine kinase 2) variants were also associated with higher interferon production, SLE and discoid and subacute lupus [47, 63].

NFκB Pathway

The NFκB pathway is triggered by multiple stimuli, including TLR activation. Several genes that participate in NFκB signaling were associated with SLE risk, namely *IRAK1* (interleukin-1 receptor associated kinase 1) [64, 65], *TNFAIP3 (*Tumor necrosis factor, alpha-induced protein 3) [3, 6, 66], *TNIP1* (TNFAIP3 Interacting Protein 1) [6, 58], *SLC15A4* (Solute Carrier Family 15 Member 4) [6] and *PRKCB* (Protein Kinase C, Beta) [67].

IRAK1 is involved in α-interferon and γ-interferon induction and is a central regulator of NFκB pathway. Five SNPs spanning *IRAK1*, an X chromosome-encoded gene, were associated with both adult- and childhood-onset SLE, in four different ethnic groups [64].

TNFAIP3 encodes A20, an ubiquitin-editing enzyme, which participates in the termination of NFκB signaling. *TNFAIP3* is an established susceptibility locus for SLE [68, 69]. Recently, a novel TT>A polymorphic dinucleotide was found to be associated with SLE in subjects of European and Korean ancestry [66]. This haplotype resulted in reduced *TNFAIP3* mRNA and A20 protein expression and the enzyme variant bound a nuclear protein complex, which included NFκB subunits, with reduced avidity [66]. This haplotype is, thus, associated with a decreased inhibitory activity of A20, which consequently causes an activation of the NFκB

pathway. The role of A20 in NFκB inhibition has been demonstrated in animal models by the development of systemic organ inflammation and death within six weeks of birth in A20 deficient mice [70], and by the existence of a lupus-like phenotype in mice with B lymphocyte specific A20 deletion [71].

Function of Monocytes and Neutrophils

The role of innate immunity in SLE has been increasingly appreciated. Monocytes play essential roles in SLE pathogenesis, since they participate in lupus nephritis and atherosclerosis, processes responsible for considerable morbidity and mortality in SLE. Increased interest in neutrophils also arose with the description of NETosis, the process by which neutrophils extrude fibrillary networks composed of DNA, histones and granular antimicrobial proteins. These NETs trap microorganisms, decreasing their ability to spread, facilitate the interaction with neutrophil-derived effector molecules and induce the production of cytokines, such as α-interferon. A positive feedback loop occurs, since this cytokine increases NETosis. In SLE, circulating immune complexes activate neutrophils and lead to an increase in the production of NETs. The DNA present in the NETs is protected from nuclease degradation, functioning as autoantigen and potentiating autoimmunity and chronic inflammation.

Genes coding for proteins related to adhesion and migration of both monocytes and neutrophils have been associated with SLE. ITGAM (CD11b), a protein mainly expressed by macrophages, monocytes and neutrophils, encodes a leucocyte-specific integrin, important in the adherence of neutrophils and monocytes to stimulated endothelium. This receptor also participates in the phagocytosis of complement coated particles and immune complexes, since it is a receptor for iC3b. An association between *ITGAM* variants and SLE susceptibility has been documented in multiple populations [4, 5, 7•, 72, 73].

B-cell Function

One of the hallmarks of SLE is the production of autoantibodies and the formation of immune complexes that drive the systemic inflammatory response. B-cells are thus key players in the pathogenesis of this disease and the existence of effective drugs that target their function, as anti-BLyS (B lymphocyte stimulator) and rituximab (anti CD-20), further supports their role in SLE. Numerous genes associated with B-cell function and signaling have been found to predispose to SLE [74•], including *BLK* (B lymphoid tyrosine kinase) [4–6], *BANK1* (B-cell scaffold protein with ankyrin repeats gene) [7•, 75] and *LYN* (tyrosine protein kinase Lyn) [5, 76], whose proteins participate in B-cell receptor signaling. The SLE-risk variants found for *BANK1* affect the regulatory sites and functional domains of the protein and contribute to sustained B-cell activation through a change in the intracellular calcium levels [75]. LYN, a src-tyrosine kinase, is a binding partner of BANK1, whose variants were also associated with SLE in European-derived individuals, with rs6983130 described as a SLE protective factor [76]. The complement receptor 2 (CR2/CD21) is a membrane glycoprotein, mainly expressed on B-cells and follicular dendritic cells, that has also been implicated in the tolerance to nuclear self-antigens such as single and double stranded DNA, chromatin and histones [77]. Reduced levels of CR2 have been described in SLE and family-based analysis provided evidence for an association of SNPs in *CR2* and SLE in Caucasian and Chinese populations [78]. This association was later confirmed in a case–control study of a European-derived population [79]. NCF2, a cytosolic subunit of the NADPH oxidase, was found to participate in B-cell activation and recently it was also implicated in SLE susceptibility [44, 58]. IL-10 is a pivotal cytokine, responsible for globally down-regulating the immune response. Interestingly, IL-10 production by monocytes and B-cells has been shown to correlate with disease activity in SLE. IL-10 polymorphisms were found to be associated with SLE in multiple populations, including European and Asian [80, 81]. IKZF1 (IKAROS family zinc finger 1) is a transcription factor involved in the regulation of lymphocyte differentiation and proliferation, and B-cell receptor signaling. It also participates in the control of *STAT4* (Signal Transducer And Activator Of Transcription 4) gene expression. Interestingly, the levels of IKZF1 were found to be decreased in the serum of SLE patients and, recently, a GWAS identified variants of *IKZF1* associated with SLE in an Asian population [6] .

T-cell Function

The role of T-cells in the orchestration of the immune response cannot be overstated, so, as expected, several genes implicated in T-cell function have also been associated with SLE, including *PTPN22* (Protein phosphatase nonreceptor type 22), *TNFSF4* (Tumor Necrosis Factor (Ligand) Superfamily, Member 4), *STAT4* and *CD247*.

PTPN22 participates in the T-cell receptor signaling pathway. A *PTPN22* SNP (rs2476601) was associated with multiple autoimmune diseases, including SLE [82]. This association was shown in a GWAS [5] and verified in a replication study [58].

TNFSF4 is a co-stimulatory molecule found on the surface of antigen-presenting cells. It binds to the T-cell receptor OX40, contributing to the global activation of T-cells, with the exception of regulatory T-cells, whose generation and function is inhibited by this signal. Protective and risk haplotypes of *TNFSF4* have been reported for SLE [83].

STAT4 is a key regulator of IL-12, IL-17, IL-23 and α-interferon signaling, having, therefore, a critical role in the development of Th1 and Th17 immune responses. Associations with SLE and multiple SNPs located within *STAT4* gene have

been found in different ethnicities, including African Americans, Hispanics and Asians [4–6, 7•, 84, 85]. There is also evidence of an association with other autoimmune diseases [85].

CD247 is a component of the T-cell receptor—CD3 complex, which was found to be decreased in SLE. Aberrant *CD247* transcript variants were detected in SLE T-cells and an association between a *CD247* SNP and SLE was detected on a recent GWAS [86].

Table 1 provides a comprehensive list of variants associated with SLE susceptibility, according to the proposed mechanism of action.

Genetic Susceptibility for SLE and other Autoimmune Diseases

The clustering of multiple autoimmune disorders in families, in addition to the identification of variants associated with increased susceptibility for different diseases, created the notion of a common autoimmunity-related genetic background. *PTPN2* is one of those examples, since variants of this gene have been associated with juvenile idiopathic arthritis, rheumatoid arthritis, systemic sclerosis, generalized vitiligo, alopecia areata, type 1 diabetes, Graves disease, Hashimoto thyroiditis, myasthenia gravis and Addison disease [2]. PS Ramos and collaborators, however, showed that only a partial pleiotropy exists among autoimmune diseases [87]. For instance, genes like *ITGAM* and *TNFSF4*, which have been clearly associated with SLE, were not found to be associated with other autoimmune diseases, and the opposite was found for *IL23R*, one of the loci found to be shared among the highest number of autoimmune diseases, but not SLE. Thus, SLE seems to have a distinct pattern of genetic susceptibility.

Table 1 List of genes whose variants were associated with SLE susceptibility

Pathway	Genes
Function of Immune Cells	
Monocytes and Neutrophils	*FCGR2B, FCGR3A/B, ICAMs, IL10, IRF8, ITGAM.*
B-cells	*AFF1, BANK1, BLK, ETS1, FCGR2B, HLA-DR2, HLA-DR3, IKZF1, IL10, IL21, IRF8, LYN, MSH5, NCF2, PRDM1, PRKCB, RASGRP3.*
T-cells	*AFF1, CD44, CD247, ETS1, FYB, HLA-DR2, HLA-DR3, IKZF1, IL10, IL21, PRDM1, PTPN22, STAT4, TNFSF4, TYK2, UBASH3A.*
Signaling	
Toll-like receptor and α-Interferon signaling	*ACP5, ELF1, ETS1, IFIH1, IRAK1, IRF5, IRF7/PHRF1, IRF8, PRDM1, STAT4, TLR7, TREX1, TYK2, UBE2L3.*
NFkB signaling	*IRAK1, PRKCB, SLC15A4, TNFAIP3, TNIP1, UBE2L3.*
Other pathways	
Clearance of immune complexes	*C1Q, C1R/C1S, C2, C4A/B, FCGR2A/B, FCGR3A/B, ITGAM.*
Apoptosis and clearance of cellular debris	*ACP5, ATG5, DNASE1, DNASE1L3, FCGR2B, TREX1.*
Production or regulation of reactive oxygen and nitrogen intermediates	*GSR, NDUFS4, NOS1.*
Loci with unknown function	*CLEC16A, JAZF1, PTTG1, PXK, TMEM39A, TNXB, UHRF1BP1, WDFY4, XKR6.*

Adapted from Rullo and Tsao [100]

The Role of Epigenetics in SLE

The phenotype of a cell is broadly determined by the epigenomic landscape, which modulates gene expression and may serve to perpetuate pathologic mechanisms. The epigenetic changes, including histone modifications, DNA methylation, and the microRNA pattern, globally determine the set of transcribed and repressed genes. DNA methylation and histone modifications change the chromatin structure to allow or prevent the access of the transcription machinery to DNA. microRNAs are non-coding RNAs responsible for post-transcriptional gene silencing, by blocking the translation or causing mRNA degradation. These regulatory molecules are involved in essential cell mechanisms, including proliferation, differentiation and apoptosis. microRNAs also exert control on the immune system, particularly on the maintenance of immunological tolerance, participating in the regulation of T-cell selection in the thymus, B-cell selection in germinal centers, and development of regulatory T-cells.

Epigenetic mechanisms are particularly important for autoimmunity, since the expression of pro-inflammatory genes, like TNF-α, is regulated at the level of the chromatin [88].

A very well characterized epigenetic change seen in SLE is the hypomethylation of DNA in T-cells, causing a state of euchromatin and, consequently, a global activation of transcription, which correlates with disease activity [89•]. Interestingly, procainamide and hydralazine, which induce lupus-like syndromes, were both found to inhibit DNA methyltransferase 1, the former directly and the latter through the inhibition of the ERK (extracellular-signal, regulated kinase) pathway [90]. Recently, a genome-wide DNA methylation study of naïve CD4+ T-cells from SLE patients and controls found significant hypomethylation in interferon-regulated genes [91]. Hypomethylation is, therefore, another mechanism responsible for the characteristic type-I interferon hyper-responsiveness seen in lupus T-cells.

Histone acetyltransferases and deacetytransferases also control gene expression by adding or removing acetyl groups on histone lysine residues. H4 acetylation is a histone modification associated with activation of transcription. This epigenomic mechanism was found to be overall increased in monocytes from SLE patients [92]. Notably, 63 % of the genes with a higher H4 acetylation had the potential of IRF1 regulation. IRF1 is an interferon-induced weak transcription factor, which regulates the transcription of genes involved in immune modulation. Interestingly, IRF1 can interact with p300 to acetylate histones, which could explain the globally increased H4 acetylation pattern seen in SLE.

MicroRNAs are also dysregulated in SLE [93•]. miR-146a, which inhibits type I interferon expression by targeting *IRF5* and *STAT-1* mRNA [94], was found to be decreased in SLE [94], contributing, therefore, for the high levels of type I interferon characteristic of this disease. Another example is miR-3148, which was found to modulate the allelic expression of a *TLR7* variant associated with SLE [95]. Finally, in a recent study a four-miRNA SLE signature was identified in plasma [96].

The interactions and consequences of these mechanisms are under intense study. Histone modifications and DNA methylation can regulate the expression of microRNAs in SLE, as is the case of miR-142 expression on T-cells from lupus patients [97], while microRNAs, like miR-21 and miR-148, which are increased in T-cells from SLE patients, decrease the expression of DNA methyltransferase 1 [98]. These findings suggest that the epigenome is globally affected in SLE and that the persistence of the epigenomic changes could lead to a durably aberrant gene expression, contributing to the perpetuation of the disease mechanisms.

Limitations of the Current Methodologies

GWAS use a high throughput technology to analyze hundreds of SNPs and capture genome common variants. Through this approach, the joint effect of many weakly contributing variants across different loci can be studied and gene variants associated with different complex diseases can be identified. This type of study is particularly tailored for complex polygenic associations, being drastically more sensitive than family studies. In comparison to linkage analysis and sequencing, however, GWAS have less power in cases of allelic heterogeneity and may be affected by the occurrence of epistasis. The majority of the variants associated with SLE susceptibility only cause a modest increase on the risk, so large sample sizes are necessary to find significant variations. Futhermore, since the loci found by this kind of study have a weak additive predictive power for a specific phenotype, their clinic relevance may be small. Finally, occasionally results from GWAS are not replicated across studies and in different populations.

Meta-analyses are an important tool to increase the statistical power and analyze the effect of gene variations across groups of different ancestries. Predictive mathematical models integrating the weakly contributing loci may also be helpful. In addition, it is necessary to understand how specific genetic variants are responsible for the association and the biological effect. Finally, fine mapping and resequencing studies are under way, as well as new tools for the analysis of transcriptomics, proteomics and metabolomics [99•], with the final goal of being able to risk-stratify patents to truly develop a personalized approach to care.

Conclusions

For most patients the pattern of SLE heritability is not characterized by a single gene with a causal Mendelian effect, but by a multigenic mode of inheritance. Further studies are necessary to understand how the identified susceptibility variants contribute to SLE manifestations. Moreover, the majority of the large-scale studies on SLE genetics were performed in European and Asian populations. Since SLE is more frequent and more severe in other groups, namely Hispanic and African-American, new studies focusing on these populations are essential. The trajectory of our understanding of the disease pathogenesis has been extraordinarily rapid since the introduction of arrays, genomic approaches and epigenetic strategies. Next generation sequencing efforts and other new technologies are also likely to rapidly advance our knowledge. The era of personalized medicine with genomic data incorporated into diagnosis, prognosis, treatment, and adverse event prevention may truly be beginning.

Compliance with Ethics Guidelines

Conflict of Interest Kathleen E. Sullivan has received gifts from CSL Behring, has received grant support from Baxter, has received honoraria from Boston Children's Hospital, and has received royalties from UpToDate.
Patrícia Costa-Reis declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of importance

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Appendix A.2 – Paper ***Major Infections in a cohort of 120 patients with Juvenile-onset Systemic Lupus Erythematosus*,** published in *Clinical Immunology*, December 2013.

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Major infections in a cohort of 120 patients with juvenile-onset systemic lupus erythematosus

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Lupus;
Infections

Abstract In order to describe the incidence and characteristics of major infections in juvenile-onset systemic lupus erythematosus (jSLE), we studied a cohort of 120 patients (51% Hispanic and 28% African American, 49% with renal involvement and 12% with neuropsychiatric manifestations). There were 101 major infections affecting 44 patients (37%) for an incidence of 169/1000 patient-years of follow-up. Active disease at jSLE diagnosis, renal involvement, neuropsychiatric manifestations, higher cumulative dose of prednisone, and treatment with cyclophosphamide or mycophenolate mofetil were all associated with major infection ($p < 0.05$). By logistic regression, the combined effect of treatment with cyclophosphamide and cumulative dose of prednisone was associated with major infection ($p = 0.04$). Two patients died, one due to cytomegalovirus infection. Major infection was associated with damage ($p = 0.004$). In conclusion, in a large cohort of jSLE patients, major infections were common, were associated with active disease and its treatment, and resulted in noteworthy morbidity.

1. Introduction

In comparison with the general population, patients with systemic lupus erythematosus (SLE) have an increased death rate from infection [1]. In a multicenter study of 1000 SLE

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patients followed over 10 years, infections were the cause of death in 25% of cases [2], with similar results observed in other studies [1,3]. Infections and active disease are responsible for the majority of deaths within the first 5 years, while myocardial infarction and thrombotic events tend to occur later, leading to a bimodal distribution of the causes of death in SLE [2]. Infections are also responsible for significant morbidity, resulting in hospitalization in 15% of patients [1]. The impact of infection on juvenile-onset SLE (jSLE) would be expected to be even higher, inasmuch as children are especially prone to infections and often have a more aggressive course of SLE requiring more potent immunosuppression [4].

Defects in innate and adaptive immunity contribute to the intrinsic risk of infection that occurs in SLE patients [5]. Immunosuppressive agents, which have become the standard for the treatment of major organ involvement in SLE, also increase this risk. Moreover, it has been reported that recurrent major infections predict poorer disease outcome in jSLE [6]. Prevention and careful monitoring for infections are thus major challenges in managing patients with jSLE.

Clinical features and treatment-related factors predisposing jSLE patients to major infection have not been adequately delineated. Furthermore, therapy for patients with jSLE has recently shifted to include increased use of steroid-sparing agents, such as mycophenolate mofetil and biological agents. New studies are, therefore, needed to clarify the risks of infections associated with different immunosuppressive regimens. The primary goal of this study was to describe the incidence and characteristics of major infections in a cohort of patients with jSLE. Our secondary aim was to identify factors, at the time of diagnosis or during the course of the disease, associated with major infections.

2. Methods

2.1. Study design and patients

A retrospective chart review was performed of the clinical courses of jSLE patients from the time of diagnosis or first encounter until December 2009 or loss to follow-up (1991–2009). The inclusion criteria were: diagnosis of SLE before the age of 18, disease duration $\geq$6 months and, at least three clinical encounters during the period 2007 to 2009 in the Pediatric Rheumatology Division of New York-Presbyterian/Morgan Stanley Children's Hospital — Columbia University Medical Center.

A diagnosis of SLE was defined by the presence of four or more of the 1997 revised American College of Rheumatology criteria for the classification of SLE [7] or when a diagnosis of lupus nephritis was confirmed by renal biopsy [8].

Demographic and clinical information were collected, including: gender; ethnicity; age at disease diagnosis; disease duration; duration of follow-up; involvement and timing of renal and neuropsychiatric manifestations; co-morbidities and treatment strategies employed. Renal lesions were categorized according to the WHO classification system for lupus nephritis [8]. Lupus activity was assessed, using the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of SLE Disease Activity Index (SLEDAI) [9].

The Systemic Lupus International Collaborating Clinics (SLICC) Damage Index (SDI) score was calculated at the conclusion of the study [10,11]. The SDI measures irreversible changes secondary to inflammation, adverse effects of medication and co-morbid conditions, which occurred since the onset of SLE, and are present for at least 6 months. Patients who died were excluded from the analysis of damage.

The Columbia University Medical Center Institutional Review Board granted ethical approval for this study.

2.2. Definition of major infections

Major infections were defined as those requiring treatment with parenteral antimicrobial agents or an oral course lasting one week or more, as previously described [6,12,13]. To diagnose an infection, it was necessary to identify a causative agent, or to have a diagnosis made by a pediatric infectious disease specialist, pediatric rheumatologist, or other attending pediatrician based on clinical findings, pathologic, and/or radiologic results.

2.3. Statistical analysis

Potential factors associated with major infection were studied, using the Chi-square test and the Mann–Whitney U test. In order to account for the effect of multiple comparisons, the Holm–Bonferroni correction was applied. Multivariate logistic regression was performed using Forward Stepwise Likelihood Ratio (LR) and Enter methods to identify possible confounding or effect-modifying factors, and to estimate adjusted odds ratios (OR) and 95% confidence intervals (95% C.I.). Time free of major infection according to jSLE disease activity and treatment was compared with actuarial analysis and expressed graphically by Kaplan–Meier survival curves. The paired t-test was used to detect differences in SLE activity during and after an infection. p-Values less than 0.05 were considered significant. Statistical analysis was performed with IBM SPSS Statistics Version 20.0® (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Demographic data

During the study period, 120 patients with jSLE fulfilled the eligibility criteria. The demographic and clinical characteristics of the study population are shown in Table 1.

The patients were mainly Hispanic (61; 51%) and African-American (34; 28%). The female to male ratio was 3.4:1. Most (63; 52%) were diagnosed between the ages of 10 and 15 years and 22 (18%) were diagnosed before 10 years of age. A family history of autoimmune diseases was found in 47 patients (39%).

The mean duration of disease at the end of the study period was 5.3 ± 3.2 years (range 6 months–17 years), while the mean duration of follow-up was 5.0 ± 3.2 years (range 6 months–17 years).

Table 1 Demographic data and clinical characteristics of jSLE patients.

	Number (%) or mean ± S.D.
Gender	
Female	93 (78%)
Male	27 (22%)
Ethnicity	
Hispanic	61 (51%)
African-American	34 (28%)
Caucasian	16 (13%)
Other	9 (8%)
Age (years) at jSLE diagnosis	
<5	5 (4%)
$5 \leq$ Age < 10	17 (14%)
$10 \leq$ Age < 15	63 (52%)
≥ 15	35 (29%)
Disease activity	
SLEDAI at diagnosis	9.6 ± 6.5
SLEDAI at time of major infection	8.2 ± 5.6
SLEDAI 6 months after a major infection episode	5.8 ± 5.7
Disease manifestations	
Leukopenia at diagnosis	8 (7%)
Lymphopenia at diagnosis	77 (64%)
Lupus nephritis	59 (49%)
Neuropsychiatric lupus	15 (12%)
Cerebrovascular accident	6 (5%)
Pericarditis	17 (14%)
Pleuritis	9 (8%)
Pulmonary hemorrhage	4 (3%)
Persistent thrombocytopenia	30 (25%)
Macrophage activation syndrome	2 (2%)
Treatment (ever use of)	
Hydroxychloroquine	120 (100%)
Mycophenolate mofetil	67 (56%)
Azathioprine	55 (46%)
Cyclophosphamide	44 (37%)
Rituximab	6 (5%)
Plasmapheresis	4 (3%)
Cumulative dose of prednisone (g)	14.4 ± 18.6

3.2. Clinical course of jSLE

At time of diagnosis, the mean SLEDAI was 9.6 ± 6.5, 7% of the patients had leukopenia (white blood cells $<3.0 \times 10^9$/L) and 64% had lymphopenia (lymphocytes $<1.5 \times 10^9$/L).

During the course of the disease, lupus nephritis was diagnosed in 59 patients (49%), of whom 48 (81%) had confirmation by biopsy; 35 (73%) had class IV lupus nephritis and 3 patients developed end-stage renal disease.

Neuropsychiatric manifestations (NPSLE) occurred in 15 (12%) patients. Cognitive dysfunction was the most common manifestation, followed by significant headache, seizures, psychosis, and focal neurologic signs. NPSLE was diagnosed in the first year of the disease in 10 patients (67%). Abnormalities on brain magnetic resonance imaging were documented in 10 patients (67%). Cerebrovascular accidents occurred in 6 patients (5%).

All patients in this cohort were treated with hydroxychloroquine. The mean cumulative prednisone dose was

Table 2 Major infections and causative organisms identified.

Types of major infection	Number	Causative organisms (n)
Skin and soft tissue infections	19	*Staphylococcus aureus* 3 Unidentified 16
Pneumonia	17	*S. aureus* 1 *Citrobacter koseri* 1 *Prevotella melaninogenica* 1 *Aspergillus fumigatus* 1 *Candida albicans* 1 Unidentified 12
Viral infections	17	Herpes simplex 4 Herpes zoster 8 Cytomegalovirus 1 Influenza 2 Mumps 1 Unidentified 1
Urinary tract infections with fever	13	*Escherichia coli* 6 *Pseudomonas aeruginosa* 2 *Proteus mirabilis* 1 *Klebsiella oxytoca* 1 *Enterococcus faecalis* 1 Unidentified 2
Ear, nose and throat infections	9	
Sinusitis	3	*Streptococcus pneumoniae* 1 Unidentified 2
Otomastoiditis	3	Unidentified 3
Retropharyngeal abscess	1	*Streptococcus pyogenes* 1
Dental abscess	2	Unidentified 2
Sepsis	10	*S. aureus* 1 *Staphylococcus capitis* 1 *S. pneumoniae* 1 *Salmonella enteritidis* 1 *Klebsiella oxytoca* 1 *Diphtheroids* 1 *C. albicans* 1 Unidentified 3
Fever and neutropenia	5	*Enterobacter cloacae 1* Unidentified 4
Gastrointestinal infections	4	
Acute gastroenteritis	3	*S. enteritidis* 2 Unidentified 1
Spontaneous peritonitis	1	Unidentified 1
Other infections [a]	7	

[a] Other infections: 3 cases of cervical adenitis; 1 case of orbital cellulitis; 1 case of sacroiliitis without identified pathogens; 1 case of meningitis caused by *S. pneumoniae*; 1 case of endocarditis caused by *Serratia marcescens*.

14.4 ± 18.6 g (range: 0–126 g). Mycophenolate mofetil (MMF) was used in 67 (56%), azathioprine in 55 (46%), cyclophosphamide in 44 patients (37%), and rituximab in 6 (5%). Four patients (3%) required plasmapheresis.

Two patients died during the study. The causes of death were disseminated cytomegalovirus infection in one patient and macrophage activation syndrome in the other.

3.3. Frequency and types of infections

During the study period, 101 major infections were diagnosed, affecting 44 patients (37%), as shown in Table 2. Twenty-three patients (19%) had recurrent episodes of major infections (≥2 episodes). The annual incidence of major infection was 169/1000 patient-years of follow-up (C.I. 95% = 139/1000–205/1000). Major infection occurred on average 4.3 ± 3.1 years after jSLE diagnosis.

The most common infections were skin and soft tissue infections (n = 19 episodes), pneumonias (n = 17), urinary tract infections with fever (n = 13), sepsis (n = 10), and herpes zoster (n = 8). The causal agent of infection was established in 49 cases (48%). The majority of the cases of herpes zoster was localized and did not require hospitalization. No cases of *Pneumocystis jiroveci* pneumonia were observed in our cohort.

The SLEDAI at time of infection (8.2 ± 5.6) was significantly higher than that 6 months following (5.8 ± 5.7) the episode (mean difference 2.4, 95% C.I. 0.8–4; p = 0.004). The median SLEDAI at the time of infection with herpes zoster was 4 and only one patient with zoster had a SLEDAI ≥8.

Leukopenia (white blood cells $<3.0 \times 10^9$/L) was present at time of major infection in 14% of episodes, lymphopenia (lymphocytes $<1.5 \times 10^9$/L) in 73% and neutropenia (neutrophils $<1.0 \times 10^9$/L) in 2%.

3.4. Factors associated with major infection

Major infection was significantly more likely to occur in patients with more active disease. A complement component 3 (C3) level <90 mg/dl at time of diagnosis was associated with major infection, as shown in Table 3. We also found an association between a higher SLEDAI at jSLE diagnosis and major infection, as shown in Table 4. No association was found between leukopenia or lymphopenia at diagnosis and major infection (data not shown).

Disease activity was important not only at diagnosis, but also during the course of SLE, since both renal involvement and neuropsychiatric manifestations of SLE were found to be associated with major infection (Table 3).

Uses of cyclophosphamide and mycophenolate mofetil were each associated with major infection in our cohort (Table 3). In order to reduce the confounding effect of treatment with both medications, we also analyzed the effect of mycophenolate mofetil in the subgroup of patients who had never received cyclophosphamide. In this group as well, treatment with mycophenolate mofetil was associated with major infection (OR 4.0; 95% C.I. 1.4–12.0; p = 0.01). The association between mycophenolate mofetil and major infection remained statistically significant even after adjusting for the effect of the cumulative dose of prednisone and disease duration (Table 3). Finally, the duration of treatment with mycophenolate mofetil was also associated with major infection, with the risk increasing 16% (OR 1.2; 95% C.I. 1.0–1.3) after 6 months and 34% after 12 months of treatment with this drug (OR 1.3; 95% C.I. 1.1–1.6). On the other hand, neither treatment with azathioprine (Table 3) nor its duration of administration increased the occurrence of major infections significantly (Table 4).

Table 3 Association between categorical variables and major infection in jSLE patients.

Variable	Proportion of major infections n/N (%)		Crude OR (95% C.I.)	p-Value	OR adjusted to cumulative prednisone dose (95% C.I.)	p-Value	OR adjusted to disease duration (95% C.I.)	p-Value
Sex	Male: 8/27 (29.6%)	Female: 36/93 (38.7%)	1.5 (0.6–3.8)	0.389	1.3 (0.4–4.3)	0.634	1.4 (0.5–3.6)	0.501
Age at diagnosis	<10 yr: 10/22 (45.4%)	≥10 yr: 34/98 (34.7%)	1.6 (0.6–4.0)	0.344	1.0 (0.3–4.0)	0.935	1.1 (0.4–3.1)	0.806
C3 at diagnosis	C3 <90 mg/dl: 26/56 (46.4%)	C3 ≥90 mg/dl: 8/39 (20.5%)	3.4 (1.3–8.6)	0.011 *	3.0 (0.9–10.0)	0.072	2.8 (1.1–7.5)	0.033
Lupus nephritis (LN)	No LN: 14/61 (23.0%)	LN: 30/59 (50.8%)	3.5 (1.6–7.6)	0.004 *	2.2 (0.8–5.7)	0.108	3.0 (1.3–6.7)	0.008
Neuropsychiatric lupus (NPSLE)	No NPSLE: 32/105 (30.5%)	NPSLE: 12/15 (80.0%)	9.1 (2.4–34.6)	0.004 *	5.9 (1.0–34.2)	0.046	8.5 (2.2–32.6)	0.002
Cyclophosphamide (CTX)	No CTX: 19/76 (25.0%)	CTX: 25/44 (56.8%)	3.9 (1.8–8.7)	0.005 *	2.5 (0.9–6.8)	0.071	3.3 (1.4–7.5)	0.005
Mycophenolate mofetil (MMF)	No MMF: 11/53 (20.8%)	MMF: 33/67 (49.2%)	3.7 (1.6–8.4)	0.003 *	2.9 (1.0–8.3)	0.041	3.5 (1.5–8.0)	0.004
Azathioprine (AZT)	No AZT: 20/65 (30.8%)	AZT: 24/55 (43.6%)	1.7 (0.8–3.7)	0.145	0.9 (0.3–2.6)	0.835	1.6 (0.7–3.4)	0.245

* p-Values corrected according to the Holm–Bonferroni method. Chi-square tests were used to study the association between variables. Logistic regression was used to determine the adjusted odds ratios. yr — years. OR - Odds Ratio.

Table 4 Association between continuous variables and infection in jSLE patients.

Factor	No infections	Infections	p-Value	p-Value adjusted to the cumulative dose of prednisone	p-Value adjusted to the disease duration
SLEDAI at diagnosis	8.2 ± 5.9	12.0 ± 6.9	0.006 *	0.242	0.012
MMF duration of treatment (months)	10.6 ± 20.4	24.3 ± 27.4	0.005 *	0.071	0.039
AZT duration of treatment (months)	5.2 ± 10.0	7.7 ± 13.6	0.246	0.764	0.348
Cumulative dose of prednisone (g)	11.0 ± 14.6	21.7 ± 23.9	0.004 *	N/A	0.011

Mann–Whitney U test was used to study the association between variables. Logistic regression was the method performed to calculate the confounding effect of cumulative dose of prednisone and disease duration. N/A - Non applicable.
* p-Values corrected according to the Holm–Bonferroni method.

Use of cyclophosphamide was associated with the occurrence of more severe infections, including sepsis (OR = 7.0; 95% C.I. 1.4–35.4; p = 0.012) and pneumonia (OR = 4.0; 95% C.I. 1.1–14.2 p = 0.03), while use of mycophenolate mofetil was not.

While variability in use occurred in this cohort, the cumulative dose of prednisone was significantly associated with major infection (Table 4). It was estimated that a 10 g increase in the cumulative dose of prednisone caused a 40% increase in the risk of major infection (OR = 1.40; 95% C.I. 1.04–1.88).

We assessed the time free of major infection in the cohort according to disease activity, SLE manifestations, and treatment as shown in the Figure. Patients with C3 < 90 mg/dl at diagnosis or those who developed neuropsychiatric manifestations of SLE during the course of the disease had a significantly lower time free of major infection (p = 0.037; p < 0.0001, respectively). Patients treated with mycophenolate mofetil or those with a cumulative dose of prednisone at the end of the study higher than 15 g also had a significant decrease on the time free from major infection (p = 0.015, on both cases) (Fig. 1).

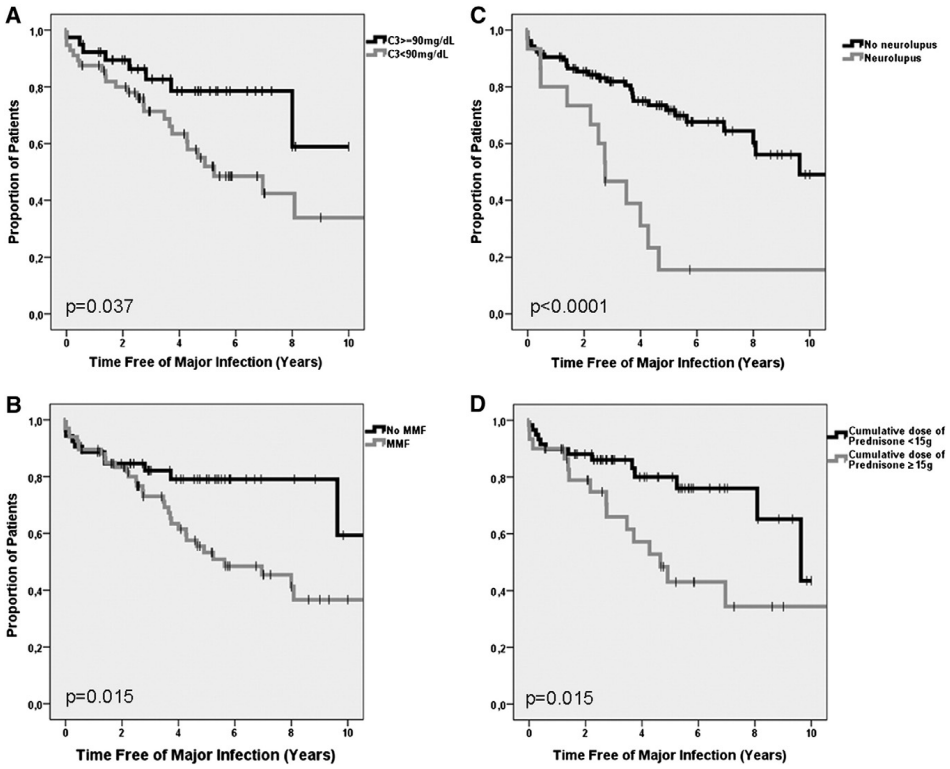

Figure 1 Kaplan–Meier analysis of time free of major infection in patients with jSLE according to disease activity and types of treatment.

The variables included in the logistic regression (Forward Stepwise Likelihood Ratio method) were history of lupus nephritis, neuropsychiatric manifestations of the disease, treatment with cyclophosphamide, treatment with mycophenolate mofetil and cumulative dose of prednisone. This analysis demonstrated that the combined effect of treatment with cyclophosphamide and cumulative dose of prednisone was significantly associated with major infection ($p = 0.04$).

3.5. Major infection and damage

Damage (SDI ≥ 1) was present at the conclusion of the study in 33% (39/118) of the surviving patients after a mean disease duration of 5.3 ± 3.2 years. Damage to the musculoskeletal system was observed most frequently (9%), followed by neuropsychiatric (7%), ocular (7%), skin (7%), pulmonary (5%) and renal (5%) systems. Avascular necrosis and osteoporosis with fracture or vertebral collapse were the most frequent types of musculoskeletal damage. No malignancies were encountered.

Major infection was associated with damage (OR = 3.2; 95% C.I. = 1.4–7.2; $p = 0.004$). This was especially true for patients with sepsis (OR = 17.1; 95% C.I. = 2.0–144.3; $p = 0.002$).

4. Discussion

The remarkable improvement in survival of jSLE patients over the past few decades, reported to be greater than 95% after 5 years of disease [6,14,15], is likely due to earlier diagnosis, better monitoring of disease activity, and more judicious use of immunosuppressive agents. In this cohort of 120 jSLE patients, 2 deaths (2%) occurred after a mean disease duration of 5.3 years.

With this increase in life expectancy, patients are faced with morbidities due to sequelae of disease and adverse effects of medication, including major infection. The focus of management has now shifted from prevention of premature death to minimizing permanent organ damage. Identification of risk factors that lead to major infection and long-term damage is therefore of increasing concern. In our cohort, a statistically significant association between major infection and damage was detected. Lee and colleagues had already shown that recurrent major infections were associated with permanent damage in jSLE [6] and Chen and coworkers also found that among SLE patients an episode of bacteremia was associated with an unfavorable long-term outcome [16].

In our study, 37% of patients had at least one episode of major infection. In a prospective and controlled study of another 110 patients with SLE and 220 controls, Bosch and colleagues found that 36% suffered at least one infection versus 22% in the control group, RR 1.63 ($p < 0.05$) [17]. Others have reported rates of infection ranging from 38% among adults with SLE [9] to 57% among children [6]. Variation in the rate of infection is most likely due to differences in age groups, duration of follow-up, disease activity, severity of organ involvement and immunosuppressive regimens employed. Furthermore, the definitions of infection have also been heterogeneous, with many studies including minor infections. Despite the variability among the criteria used, the spectrum of infections and causative microorganisms has been similar in adults and children.

Skin and soft tissue infections, pneumonias, urinary tract infections with fever, sepsis and herpes zoster were the most common infections found in our study and reported in the literature [1,6,17–19]. In addition to unusual opportunistic infections, jSLE patients also experienced infections caused by common pathogens that behaved more aggressively. For example, a case series suggested an increased risk of nontyphoidal *Salmonella* infection in SLE patients, in association with cellular immune defects [20–23]. We also documented three cases of nontyphoidal *Salmonella*, including one case of sepsis.

Herpes virus infections are also common. The reported incidence of herpes zoster in SLE ranges from 6.4 to 16 events/1000 patient-years, which is remarkably greater than the one reported in the general population [24–26]. The incidence found in our cohort was 13 events/1000 patients-years with a median SLEDAI at the time of herpes zoster of 4. These results are in concordance with other studies that showed that herpes zoster usually occurs during periods of quiescent SLE activity [26].

It has been reported that over 90% of SLE patients are seropositive for cytomegalovirus and while overt clinical disease is rare, it carries a high risk of mortality [27–29]. In the current study, we found similar results, considering that only one case of cytomegalovirus infection was identified and resulted in the death of the patient.

Infections caused by Parvovirus B19 and Epstein–Barr virus were not documented in our cohort, but potentially were underdiagnosed as these viruses were not routinely investigated.

We did not encounter any case of *P. jiroveci* infection, even though we do not routinely provide prophylaxis against this organism. Gupta et al. have also shown that the number of reported infections with this agent in SLE patients treated with cyclophosphamide is very low (15.88/10,000 patients) [30]. Our data complements, therefore, the growing body of evidence against the need for prophylaxis against *P. jiroveci* in jSLE.

Disease activity was clearly associated with the occurrence of major infection in this study, as a higher SLEDAI at diagnosis, renal involvement, and neuropsychiatric manifestations were all identified in univariate analysis as factors associated with major infection. Others have found lung and renal involvement [18] and SLEDAI >12 at diagnosis to be predictors of infection [12]. In addition, similar to our findings, low complement levels (C3 <90 mg/dl) have been documented as predictors of major infection [12,17].

In our cohort, use of cyclophosphamide was associated with major infection. Although Bosch and colleagues also documented this association [17], it has not been consistently noted by others [1,12].

We found that mycophenolate mofetil treatment was strongly associated with major infection in jSLE, even in the subgroup of patients who had never been treated with cyclophosphamide and after adjusting for the effects of cumulative dose of prednisone and disease duration. Furthermore, the duration of treatment with mycophenolate mofetil was also found to be a risk factor for major infection in the univariate analysis. On the other hand, azathioprine treatment and its duration were not associated with major infection.

The cumulative dose of prednisone was associated with major infections in our cohort as described in other series [12,17], but not all [1]. Ruiz-Irastorza and colleagues concluded that an increase of 10 mg per day of prednisone caused an 11-fold increase in the risk of serious infection [18].

In this cohort, according to multivariate analysis, the combined effect of treatment with cyclophosphamide and cumulative dose of prednisone was strongly associated with major infection, which once again supports the need of a judicious use of these drugs.

The relationship between SLE and infections is complex. Not only can active disease lead to infection, but infections can also act as environmental triggers that induce or promote SLE flares. In our cohort, we found that the disease was clearly more active at the time of infection when compared to a clinical encounter 6 months later.

This study had limitations. This was a retrospective study with the potential for missing data and lack of standardized treatment. Our center serves as a tertiary referral center, which most likely increases the severity of disease. Moreover, in our cohort the majority of the patients are Hispanic or African-American, which represent groups known to have more severe courses of jSLE, and might not be generalizable to other centers. We could not assess the effect of hydroxychloroquine on major infections, as all patients in our cohort were treated with this agent, nor could we assess the effect of rituximab due to the small number of patients treated with this agent prior to 2009. We did not include data on autoantibodies, since there was great variability in the methods used for their measurement. The retrospective nature of this study also limited the possibility of assessing for the presence of hypogammaglobulinemia at the time of infection. Finally, many variables (e.g., lupus activity, hypocomplementemia, renal disease, neuropsychiatric manifestations, treatment with corticosteroids and other immunosuppressive drugs) are interrelated in clinical practice, and their relative weight and interactions are difficult to determine, even with multivariate analysis. Including a control group of age- and sex-matched children with juvenile idiopathic arthritis might have allowed further determination of the risk of major infection due to SLE itself and what is truly related to immunosuppression.

In conclusion, in this large cohort of urban North American children and adolescents with jSLE, major infections were found to be common, were associated with active disease and its treatment, and resulted in noteworthy morbidity. In order to ensure optimal patient outcomes, it is essential, therefore to perform regular surveillance for infections, with frequent visits, particularly in patients with more active disease and undergoing treatment with immunosuppressive drugs, and to promptly initiate treatment on presumption of infection. In a recent review of patients with SLE admitted to an Intensive Care Unit, a delay in adequate antimicrobial therapy of more than 24 h was associated with increased mortality [31].

Finally, many infections can be prevented with timely immunization, reducing exposure to contagious contacts, screening for latent infection, and minimizing exposure to immunosuppressive drugs, which are essential to achieve optimal control of SLE activity, but the judicious use of which is equally important to avoid infections and other associated adverse effects [32]. These measures will help to reduce the burden of major infections in jSLE patients and consequently improve patient outcomes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A.3 – Paper ***The Role of MicroRNAs and Human Epidermal Growth Factor Receptor 2 in Proliferative Lupus Nephritis***, published in Arthritis and Rheumatology, September 2015.

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The Role of MicroRNAs and Human Epidermal Growth Factor Receptor 2 in Proliferative Lupus Nephritis

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Objective. **To understand the roles of microRNAs (miRNAs) in proliferative lupus nephritis (LN).**

Methods. **A high-throughput analysis of the miRNA pattern of the kidneys of LN patients and controls was performed by molecular digital detection. Urinary miRNAs were measured by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Target gene expression in human mesangial cells was evaluated by arrays and qRT-PCR. Human epidermal growth factor receptor 2 (HER-2) was analyzed by immunohistochemistry in kidney samples from LN patients and in a murine model of lupus. Urinary levels of HER-2, monocyte chemotactic protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) were measured by enzyme-linked immunosorbent assay.**

Results. **Levels of the miRNAs miR-26a and miR-30b were decreased in the kidneys and urine of LN patients. In vitro these miRNAs controlled mesangial cell proliferation, and their expression was regulated by HER-2. HER-2 was overexpressed in lupus-prone NZM2410 mice and in the kidneys of patients with LN, but not in other mesangioproliferative glomerulonephritides. HER-2 was found to be up-regulated by interferon-α and interferon regulatory factor 1. Urinary HER-2 was increased in LN and reflected disease activity, and its levels correlated with those of 2 other recognized LN biomarkers, MCP-1 and VCAM-1.**

Conclusion. **The kidney miRNA pattern is broadly altered in LN, which contributes to uncontrolled cell proliferation. Levels of the miRNAs miR-26a and miR-30b are decreased in the kidneys and urine of LN patients, and they directly regulate the cell cycle in mesangial cells. The levels of these miRNAs are controlled by HER-2, which is overexpressed in NZM2410 mice and in the kidneys and urine of LN patients. HER-2, miR-26a, and miR-30b are thus potential LN biomarkers, and blocking HER-2 may be a promising new strategy to decrease cell proliferation and damage in this disease.**

Lupus nephritis (LN) is associated with considerable morbidity and mortality, particularly in children (1,2). The diagnosis of LN is based on renal biopsy findings, which have a modest predictive value for outcome. Noninvasive strategies are thus needed for both the diagnosis and monitoring of LN. Furthermore, treatment of LN (3) is associated with several side effects, including major infections (4) and infertility (5). The identification of new biomarkers to guide the judicious use of these therapeutic agents and the development of new treatment strategies with fewer side effects would have an enormous impact on the management of disease in LN patients.

Cellular microRNAs (miRNAs) are noncoding RNAs with a key role in the posttranscriptional regula-

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tion of gene expression. Each miRNA can regulate hundreds of target messenger RNAs (mRNAs) and thereby control almost every biologic pathway (6). MicroRNA dysregulation can elicit a dramatic change in cell behavior, therefore contributing to the pathogenesis of human diseases. The development of high-throughput methodologies for the global measurement of miRNAs, and the stability of these molecules in biologic fluids, have allowed them to emerge as a new class of biomarkers.

Several studies of miRNAs in systemic lupus erythematosus (SLE) have been performed in plasma (7), in peripheral blood mononuclear cells (8–10), and in the kidneys of LN patients (11,12). However, no unbiased analysis of renal tissue has been performed.

We studied the miRNA-mediated mechanisms of LN and identified the human epidermal growth factor receptor 2 (HER-2) pathway as a regulator of miRNA expression and cell proliferation. Our findings suggest a model whereby type I interferons (IFNs) up-regulate HER-2 expression, which down-regulates miRNA-26a (miR-26a) and miR-30b, releasing cell cycle transcripts from repression and contributing to the mesangial cell proliferation seen in LN. These data identify a new pathway for therapeutics and also a new robust biomarker.

PATIENTS AND METHODS

Patients and kidney specimens. Paraffin-embedded kidney samples were selected from pediatric patients with LN or poststreptococcal glomerulonephritis. The controls were normal kidney samples from adult donors and 1 sample of normal kidney tissue from a child with nephroblastoma. The demographic and clinical characteristics of the LN cohort are summarized in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract. A pathologist (PAR) confirmed the diagnoses and classified the LN findings according to the International Society of Nephrology/Renal Pathology Society criteria (13). The Institutional Review Board (IRB) of The Children's Hospital of Philadelphia approved the study.

RNA extraction and miRNA quantification. RNA was extracted with an miRNeasy FFPE Kit (Qiagen) and analyzed using a NanoDrop 2000 spectrophotometer. High-purity samples were chosen, according to the absorbance ratios 260:280 and 260:230. Seven hundred thirty-four miRNAs were analyzed by direct digital detection of molecular barcodes, with an nCounter assay (NanoString). This method is ideal for fragmented RNA samples and is highly sensitive for miRNAs. Six negative miRNA assay controls, 6 positive miRNA spikes, and 5 housekeeping mRNA controls (*ACTB*, *B2M*, *GAPDH*, *RPL19*, and *RPL0*) were also quantified. The data were normalized to the sum of the 6 positive control miRNA spikes. To account for differences in miRNA content in each sample, the data were normalized to the sum of all miRNA counts for each assay. Principal components analysis (PCA) was performed using R statistical computing language (www.r-project.org), and pathway analysis was done using IPA. Statistical significance was defined as a Benjamini-Hochberg false discovery rate of <0.05.

Kidney levels of miR-26a and miR-30b were studied in the same cohort by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) using TaqMan miRNA assays (Applied Biosystems) and a 7900HT sequence detection system (Applied Biosystems). Relative quantification was applied using spiked *Caenorhabditis elegans* miR-238 as control (Qiagen). Commercially available primers were purchased from Applied Biosystems (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract).

Detection of miRNA in urine. Urine samples were collected from healthy individuals and adult patients followed up at the Hospital of the University of Pennsylvania. All of the patients had demonstrated clinical and laboratory evidence of active LN (hematuria, proteinuria, pyuria, and/or urinary casts) at 1 or more encounters in the past 3 years. Characteristics of this cohort are displayed in Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract.

Urine samples were centrifuged at 3,000 revolutions per minute for 30 minutes at 4°C, and aliquots of 100 μl of the supernatants with 500 μl of QIAzol lysis reagent were kept at −80°C. RNA was extracted from these samples using a Qiagen miRNeasy Serum/Plasma Kit. The miRNAs miR-26a and miR-30b were measured by qRT-PCR.

Knockdown and overexpression of miRNAs in human mesangial cells. Human renal mesangial cells (ScienCell) were selected for in vitro studies, since the proliferation of this type of cell is an important phenomenon of LN pathogenesis. The cells were maintained according to the manufacturer's recommendations.

Mesangial cells were transduced with lentiviruses (Applied Biological Materials) (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract) at a multiplicity of infection of 1 and in the presence of polybrene (3 μg/ml). The lentiviruses expressed miRNA inhibitors complementary to the targeted mature miRNA sequence under the control of the H1 promoter. Green fluorescent protein and the puromycin resistance gene were under the control of the cytomegalovirus (CMV) promoter. The infection efficiency was evaluated by fluorescence with an Axio Observer A1 microscope (Carl Zeiss). Stable cells with knockdown of miRNA were obtained with puromycin selection and were used as polyclonal populations. The controls were mesangial cells transduced with a lentivirus vector and uninfected cells.

RNA was extracted with a Qiagen RNeasy kit and analyzed with a 2100 Bioanalyzer (Agilent). The transcriptome was amplified with an Ovation Pico WTA System V2 (NuGEN). Whole-genome expression of the cells with knockdown of miRNA and controls was studied using Affymetrix GeneChip Human Gene 2.0 ST arrays. Gene expression data were normalized and quality controls were assessed before further analyses. Canonical pathways were studied using IPA.

A Clontech Advantage RT kit was used to generate complementary DNA for the study of transcripts. Gene expression was detected by qRT-PCR and normalized to 18S ribosomal RNA. Commercially available primers were purchased from Applied Biosystems (see Supplementary Table 2,

available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract).

Overexpression of miRNA was achieved by transfection of pCMV-MiR constructs for miR-26a and miR-30b (OriGene). These cells were maintained in 20% calf serum. Gene expression was evaluated by qRT-PCR.

Cell proliferation was analyzed using an MTT assay. The cells were seeded at a density of 100,000 cells/well, cultured for 3 days, and treated according to the protocol of the manufacturer (Sigma). The number of viable cells was calculated according to a standard curve. As a validation strategy, cell proliferation was also measured after 6 days of culture, using propidium iodide (10 mg/ml; Sigma) staining with Igepal permeabilization followed by spectrophotometric detection.

Effect of an anti–HER-2 drug in human mesangial cells. Mesangial cells were exposed to 8 μg/ml trastuzumab (kindly provided by Genentech). After 24 hours of culture, miR-26a and miR-30b were quantified by qRT-PCR, using *C elegans* miR-238 and U6 as controls. After 3 days of culture, RNA was extracted and whole-genome expression arrays were performed. The results were compared to those for mock-treated mesangial cells.

Immunohistochemistry for HER-2 in humans. Fully automated immunohistochemistry was performed on a Leica Bond-Max with rabbit anti–HER-2 (1:500, staining for 1 hour; Sigma-Aldrich) after antigen retrieval at low pH for 20 minutes. A Bond Polymer Refine Detection system (Leica Biosystems) was used. Images were obtained using an Aperio ScanScope eSlide capture device. HER-2–positive breast cancer tissue was used as a positive control. For every sample, negative staining controls were executed without the primary antibody.

Immunohistochemistry for HER-2 in a mouse model of LN. HER-2 expression was studied in NZM2410 mice (a mouse model of LN) (14) and in BALB/c and C57BL/6 (B6) mice (nonautoimmune controls). All 3 strains of mice were obtained from The Jackson Laboratory. Blood and urine were collected every other week from NZM2410 mice. The characteristics of the mice are shown in Supplementary Table 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract.

Immunohistochemistry was performed on formalin-fixed kidney tissue with rabbit anti–HER-2 (1:300, staining for 1 hour) after heat antigen retrieval with a low-pH citrate solution (Vector). Amplification was performed with Vectastain avidin–biotinylated enzyme complex and Vector biotinylated anti-rabbit antibody. Negative controls (without primary antibody) were performed in parallel. The number of HER-2–positive cells in the glomeruli of NZM2410 mice was compared to that in controls. On average, 113 glomeruli were analyzed per mouse. A blood urea nitrogen (BUN) level of $>$30 mg/dl was considered elevated. All experiments were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia.

Expression studies in vitro. Mesangial cells were treated with 100 units/ml IFNα (Biomedical Laboratories) for 3 days. HER-2 expression was evaluated by qRT-PCR and compared to that in mock-treated cells (primers are listed in Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract).

IFN regulatory factor 1 (IRF-1)–puromycin plasmids and vector-only plasmids were transfected into mesangial cells using an Amaxa Cell Line Nucleofector Kit V (Lonza). IRF-1 transfection was confirmed by qRT-PCR and also by immunofluorescence, using mouse anti-human IRF-1 (1:50, incubation for 1 hour; Santa Cruz Biotechnology) as primary antibody and Alexa Fluor 546–conjugated goat anti-mouse IgG (Invitrogen) as secondary antibody. The corrected total cell fluorescence was calculated using Image J software (National Institutes of Health). HER-2 expression was evaluated by qRT-PCR. The miRNAs miR-26a and miR-30b were also measured by qRT-PCR, using spiked *C elegans* miR-39 (Qiagen) as control.

Quantification of HER-2, monocyte chemotactic protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) in urine. Using human urine samples, enzyme-linked immunosorbent assays for HER-2 (Abcam), MCP-1 (R&D Systems), and VCAM-1 (R&D Systems) were performed according to the manufacturers' instructions. Spectrophotometry was performed with an EL808 microplate absorbance reader (BioTek Instruments).

Urine samples from LN patients were obtained from the Johns Hopkins Lupus cohort (15). Samples from patients with active LN at the time of urine collection (renal SLE Disease Activity Index [SLEDAI] [16] score of $\geq$4) were compared to samples from adult, healthy, sex- and age-matched individuals. The characteristics of the patients are shown in Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract. Samples from patients with active LN were also compared to samples from the same patients collected at least 3 months before the identified flare, when their renal SLEDAI score was zero. To assess the association between HER-2 and biopsy results, we studied samples that were collected from patients who underwent a kidney biopsy within 1 year from the date of the urine collection. The IRB offices of The Children's Hospital of Philadelphia and of the Johns Hopkins University School of Medicine approved this study. Urine samples were collected in sterile containers and a protease inhibitor was added (cOmplete Protease Inhibitor Cocktail; Roche). The samples were placed on ice or refrigerated at 4°C within 1 hour of collection and stored at −80°C. HER-2, MCP-1, and VCAM-1 values were normalized by dividing by the urine creatinine concentration.

Statistical analysis. GraphPad Prism software version 5.0 was used for the statistical analysis. Unpaired *t*-tests and Mann-Whitney U tests were used for comparisons between samples with normal and non-normal distributions, respectively. The relationship between HER-2 and the other urinary biomarkers was defined by Pearson's correlation and linear regression. *P* values less than 0.05 were considered significant. In vitro experiments were performed at least 3 times.

RESULTS

LN has a characteristic kidney miRNA signature that reflects cell proliferation. The expression of more than 700 miRNAs was analyzed in the kidneys of controls and children with LN and poststreptococcal glomerulonephritis. PCA showed that broadly the 3 groups clustered in different parts of the diagram (see Supplementary Figure 1, available on the *Arthritis & Rheumatology*

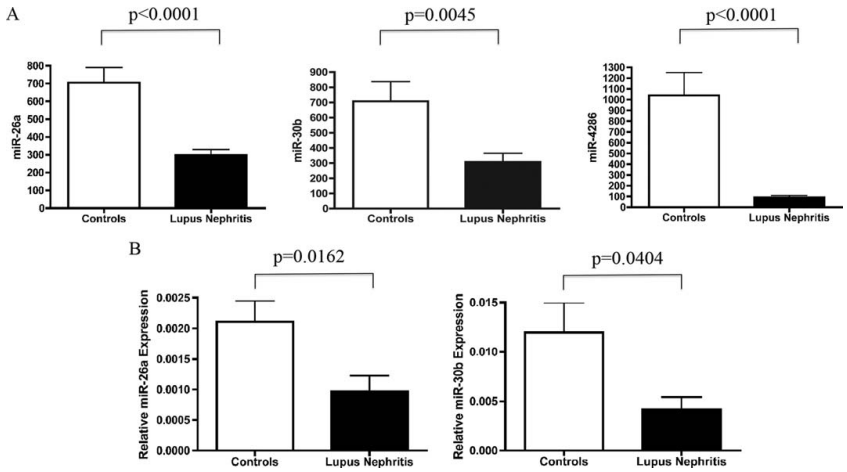

Figure 1. Levels of the microRNAs miR-26a and miR-30b are decreased in the kidneys and urine of patients with lupus nephritis (LN). **A,** Levels of miR-26a, miR-30b, and miR-4286 in the kidneys of pediatric LN patients (n = 12) and controls (n = 6) were measured by direct digital detection of molecular barcodes. **B,** Levels of miR-26a and miR-30b in the urine of adult LN patients (n = 14) and controls (n = 19) were analyzed by quantitative reverse transcription–polymerase chain reaction. Values are the mean ± SEM.

web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). No differences were seen between the different types of controls. These findings suggested that the renal LN miRNA pattern is characteristic of the disease and reflects its specific pathogenesis.

Levels of 41 miRNAs were significantly decreased in the kidneys of LN patients compared to controls ($P < 0.05$) (see Supplementary Table 6, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). According to IPA, these miRNAs were associated with cell cycle (P values of 9.02×10^{-5} to 2.26×10^{-2}). We selected 3 miRNAs for further study (miR-26a, miR-30b, and miR-4286) (Figure 1A) based on the magnitude of their differential expression and P values ($P < 0.0001$, $P = 0.0045$, and $P < 0.0001$, respectively). There were no significant differences in the expression of these miRNAs according to sex, ethnicity, age, or immunosuppressive regimen used (data not shown). Patients without previous treatment also had a statistically significant decrease in miR-26a, miR-30b, and miR-4286 compared to controls ($P = 0.0005$, $P = 0.0244$, and $P = 0.0003$, respectively). Levels of housekeeping genes were no different between LN patients and controls. The data for miR-26a and miR-30b levels were validated by qRT-PCR (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract).

We next focused our functional analyses on miR-26a and miR-30b, since miR-4286 was recently described and its cellular functions are still unknown. The fragmented RNA from the paraffin-embedded samples could not be interrogated to confirm a transcriptome signature related to dysregulation of miR-26a and miR-30b; however, we performed an in silico analysis of data available publicly (17). The transcriptome in LN glomeruli showed a significant de-repression of predicted miR-26a and miR-30b targets (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). DAVID terms enriched in the miR-26a targets up-regulated in LN glomeruli were blood pressure, transmembrane proteins, defense response, response to wounding, immune response, and lipoproteins ($P < 10^{-4}$). For miR-30b, the terms were immune response, disulfide bond, regulation of blood pressure, and regulation of proliferation ($P < 10^{-4}$). These data support the concept that miR-26a and miR-30b dysregulation is biologically relevant in LN.

Decreased levels of miRNAs miR-26a and miR-30b in the urine of LN patients. We analyzed the urinary levels of miR-26a and miR-30b to explore their potential role as LN biomarkers. We also observed decreased levels of these miRNAs in the urine of adult LN patients compared to healthy controls, as was the case in renal tissues ($P = 0.0162$ and $P = 0.0404$, respectively) (Figure 1B).

Control of cell cycle–related gene expression by miR-26a, miR-30b, and miR-4286. Cell cycle pathways were strongly enriched in our analysis of the LN miRNA

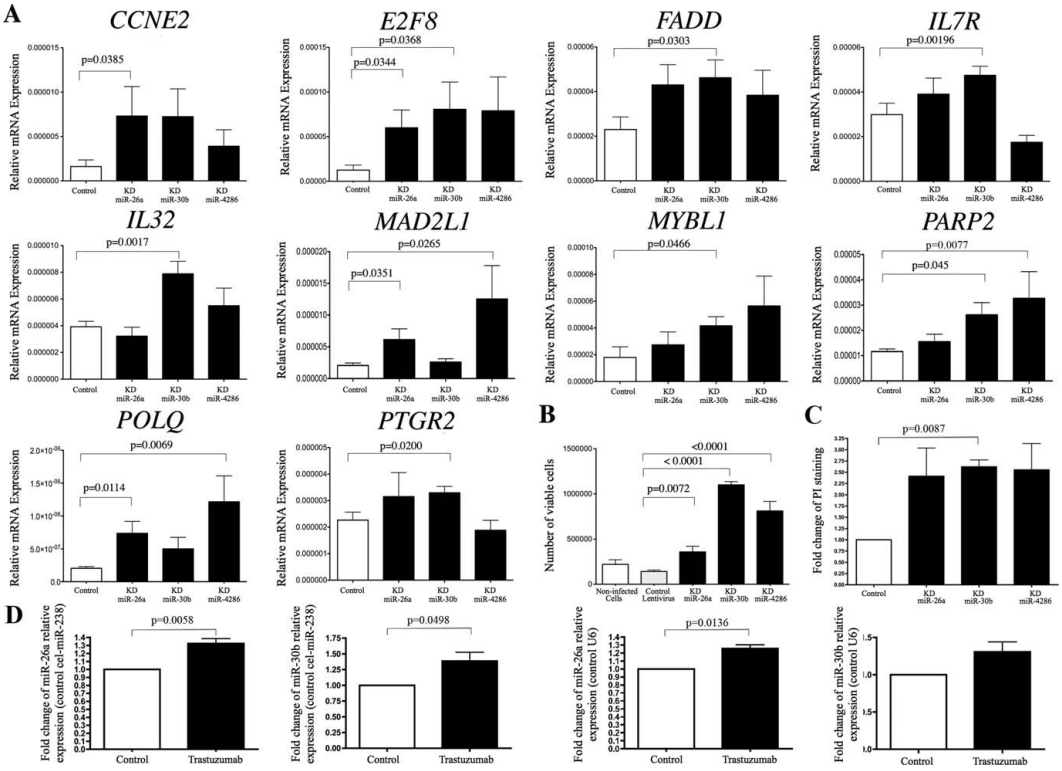

Figure 2. Mesangial cells with knockdown (KD) of microRNAs (miRNAs) miR-26a, miR-30b, or miR-4286 have higher expression of genes related to cell cycle and proliferate more. **A,** Expression of different miRNA targets in mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286 and in vector-transduced control cells was evaluated by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). **B,** MTT assays were performed to study cell proliferation in mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286, in cells transduced with a lentivirus control vector, and in uninfected cells. **C,** Mesangial cells with knockdown of miR-26a, miR-30b, or miR-4286 were cultured for 6 days and evaluated with propidium iodide (PI). **D,** Levels of miRNAs were measured by qRT-PCR after treatment with 8 μg/ml trastuzumab for 24 hours. Values are the mean ± SEM. Experiments were performed at least 3 times. cel-miR-238 = *Caenorhabditis elegans* miR-238.

signature, and proliferation is a hallmark of LN. We therefore investigated whether these miRNAs directly regulated cell cycle gene transcript abundance. We knocked down each implicated miRNA in human mesangial cells and analyzed the gene expression by arrays. According to IPA, knockdown of miR-26a led to increased expression of genes associated with cell cycle (P values of 9.98×10^{-9} to 1.98×10^{-2}) and DNA replication, recombination, and repair (P values of 1.10×10^{-8} to 1.98×10^{-2}). Similar results were obtained for miR-30b knockdown (P values of 1.47×10^{-20} to 1.11×10^{-2} for both types of genes) and miR-4286 knockdown (P values of 9.39×10^{-22} to 1.15×10^{-2} for both types of genes). These findings suggested that miR-26a, miR-30b, and miR-4286 were directly regulating the expression of genes involved in proliferation. The expression levels of 10 genes were validated by qRT-PCR (Figure 2A). Conversely, when miR-26a and miR-30b overexpression vectors were transfected into mesangial cells, we saw decreased expression of the cell cycle genes (see Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). Thus, the miRNA signature appears to be intimately involved in pathologic cell cycle gene expression in LN.

Control of cell proliferation by miR-26a, miR-30b, and miR-4286. We directly examined proliferation by performing MTT assays. A statistically significant increase in the number of viable mesangial cells was seen with knockdown of miR-26a, miR-30b, or miR-4286

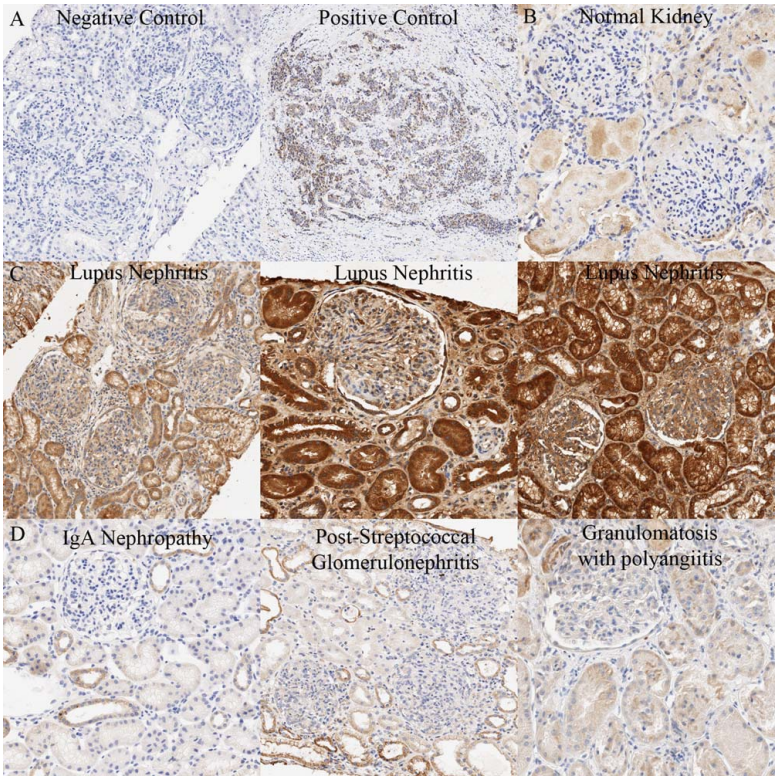

Figure 3. Human epidermal growth factor receptor 2 (HER-2) expression is increased in kidneys of patients with lupus nephritis (LN) compared to its expression in normal kidneys and in kidneys of patients with other proliferative glomerulonephritides. **A,** Positive and negative controls (with HER-2–positive breast cancer tissue and without primary antibody, respectively). **B–D,** Representative samples of kidneys from healthy donors (n = 2) (**B**), from LN patients (n = 8) (**C**), and from patients with other proliferative glomerulonephritides (IgA nephropathy [n = 4], poststreptococcal glomerulonephritis [n = 4], and granulomatosis with polyangiitis [n = 4]) (**D**). Original magnification × 100.

compared to cells transduced with the lentivirus control ($P = 0.0072$, $P < 0.0001$, and $P < 0.0001$, respectively) (Figure 2B) (see Supplementary Figure 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). These data were further confirmed using propidium iodide to measure DNA content (Figure 2C).

Increased levels of miR-26a and miR-30b induced by trastuzumab along with lowered expression of cell cyle–related genes. Trastuzumab, a monoclonal antibody that inhibits HER-2, causes arrest at the G_1 phase in breast cancer cells by increasing miR-26a and miR-30b (18). We hypothesized that trastuzumab might also increase miR-26a and miR-30b in mesangial cells and affect their cell cycle. Levels of miR-26a and miR-30b were measured in trastuzumab-treated cells and were found to be increased, using either *C elegans* miR-238 or U6 as control (Figure 2D). Furthermore, trastuzumab-treated cells had decreased expression of genes related to cell cycle (P values of 3.96×10^{-5} to 2.70×10^{-2}) and DNA replication, recombination, and repair (P values of 1.39×10^{-8} to 2.70×10^{-2}). These data confirm that in mesangial cells, similar to breast cancer cells, trastuzumab can increase miR-26a and miR-30b and inhibit the expression of cell cycle–related genes.

Dramatically increased levels of HER-2 in LN, but not in other proliferative glomerulonephritides. The effects of trastuzumab led us to hypothesize that increased HER-2 might be regulating miRNA expression in LN. No prior studies of HER-2 expression

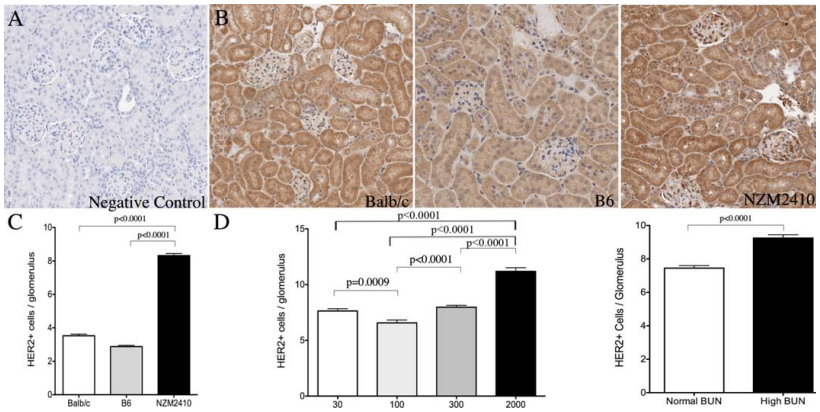

Figure 4. Significantly more human epidermal growth factor receptor 2 (HER-2)–positive cells per glomerulus detected in NZM2410 mice than in BALB/c or C57BL/6 (B6) mice. **A,** Negative control experiment performed on NZM2410 mouse kidneys without the use of the primary antibody. **B,** Immunohistochemical studies of HER-2 in the kidneys of BALB/c mice (n = 3), B6 mice (n = 10), and NZM2410 mice (n = 9). Representative results are shown. **C,** Numbers of HER-2–positive cells per glomerulus in BALB/c mice, B6 mice, and NZM2410 mice. **D,** Numbers of HER-2–positive cells per glomerulus in NZM2410 mice, according to proteinuria (mg/dl) (left) and blood urea nitrogen (BUN) level at the time that mice were killed (normal ≤30 mg/dl; high >30 mg/dl) (right). Values are the mean ± SEM. Original magnification × 40.

had been performed in this disease. We found a dramatic increase in HER-2 expression in the kidneys of LN patients, not only in the tubular compartment, but also in the glomeruli, where mesangial cells, endothelial cells, and podocytes were strongly stained. Normal kidneys and kidneys from patients with other proliferative glomerulonephritides (IgA nephropathy, poststreptococcal glomerulonephritis, and granulomatosis with polyangiitis) had light staining of tubules, but no strongly HER-2–positive cells in the glomeruli (Figure 3).

Increased HER-2 levels in the glomeruli of NZM2410 mice. To further examine HER-2 in LN, immunohistochemical studies were also conducted in NZM2410 mice and in control B6 and BALB/c mice (Figure 4). As expected, NZM2410 mice developed an early-onset, aggressive, lupus-like diffuse proliferative glomerulonephritis. While HER-2 staining was almost absent from kidneys in healthy humans, tubules in healthy B6 and BALB/c mice showed strong HER-2 staining, with glomeruli being mostly negative. However, kidneys from NZM2410 mice showed a significantly higher number of HER-2–positive cells per glomerulus compared to kidneys from B6 and BALB/c mice ($P < 0.0001$ for both) (Figure 4C). In addition, the number of HER-2–positive cells per glomerulus was significantly higher in NZM2410 mice with higher proteinuria and higher levels of BUN (Figure 4D), suggesting that HER-2 expression correlates with severity of disease.

Increased HER-2 expression induced by IFNα and IRF-1 in human mesangial cells. Our data suggested a model whereby increased HER-2 expression drives decreased miR-26a and miR-30b levels, allowing for de-repression of cell cycle transcripts. We next wished to understand the etiology of the increased HER-2 expression. Since type I IFNs have been implicated in SLE, we analyzed whether IFNα could regulate HER-2 expression. We found that mesangial cells exposed to IFNα indeed had significantly higher expression of HER-2 than did control cells ($P = 0.02$) (Figure 5D).

IRF-1 is a key transcription factor induced by IFNα. We therefore decided to examine its effect on HER-2 expression. IRF-1 transfection was confirmed by immunofluorescence and qRT-PCR, as shown in Figure 5A. HER-2 expression was significantly increased in IRF-1–transfected cells ($P = 0.0009$) (Figure 5B). Furthermore, decreased levels of miR-26a and miR-30b were seen in IRF-1–transfected cells (Figure 5C). These data suggested that lupus-associated factors such as IFNα and IRF-1 contribute to HER-2 overexpression and to secondarily decreased miR-26a and miR-30b levels seen in LN.

Increased HER-2 levels in urine of LN patients, and association of HER-2 with disease activity. The dramatic overexpression of HER-2 in LN renal tissue

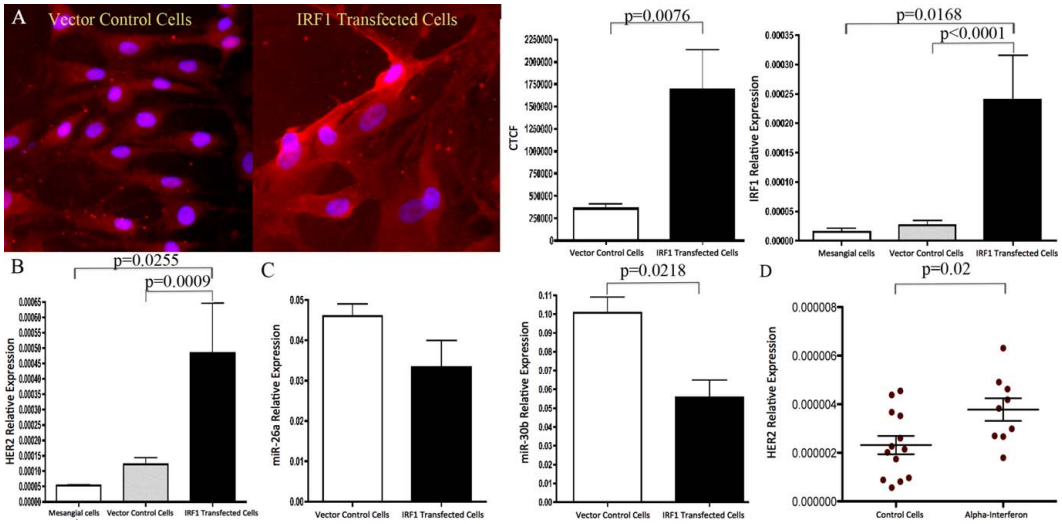

Figure 5. Interferon-α (IFNα) and IFN regulatory factor 1 (IRF-1) increase the expression of human epidermal growth factor receptor 2 (HER-2) in human mesangial cells. **A,** Successful overexpression of IRF-1 in IRF-1–transfected cells, measured by immunofluorescence (left) and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) (right). CTCF = corrected total cell fluorescence. Original magnification $\times$ 100. **B,** Expression of HER-2 in mesangial cells transfected with IRF-1 or with the control vector, measured by qRT-PCR. **C,** Levels of miR-26a and miR-30b in cells transfected with IRF-1 or with the control vector, measured by qRT-PCR. **D,** Expression of HER-2 in mesangial cells with or without previous exposure to IFNα, measured by qRT-PCR. In **A–C**, values are the mean ± SEM. In **D**, symbols represent individual samples; bars show the mean ± SEM. Experiments were performed at least 3 times.

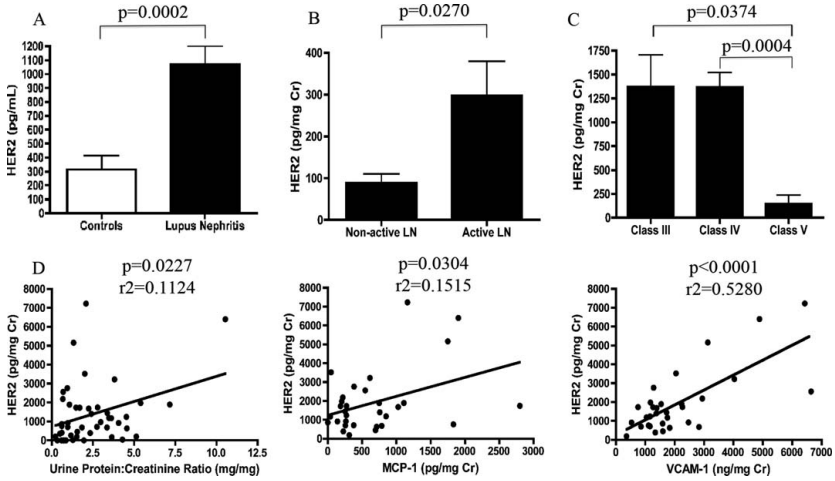

Figure 6. Human epidermal growth factor receptor 2 (HER-2) is increased in the urine of patients with active lupus nephritis (LN) and is associated with disease activity. **A,** HER-2 levels in the urine of adult patients with active LN (n = 47) and in sex- and age-matched healthy controls (n = 26) were measured by enzyme-linked immunosorbent assay. **B,** HER-2 levels were analyzed in 14 patients at 2 different time points (during inactive LN and at the time of an LN flare). **C,** Urinary HER-2 levels were analyzed according to histologic class of LN (n = 19). **D,** HER-2 levels were correlated with the urine protein:creatinine (Cr) ratio (n = 46) and with levels of monocyte chemotactic protein 1 (MCP-1) (n = 31) and vascular cell adhesion molecule 1 (VCAM-1) (n = 31). In **A–C**, values are the mean ± SEM.

suggested that it could be a useful biomarker. We found that HER-2 was significantly increased in the urine of adult patients with active LN compared to healthy controls ($P = 0.0002$) (Figure 6A). Moreover, when we analyzed HER-2 levels longitudinally, we found that they were significantly increased during LN flares ($P = 0.0270$) (Figure 6B). HER-2 was also significantly increased in the urine of patients with class III and class IV LN compared to the urine of patients with class V LN ($P = 0.0374$ and $P = 0.0004$, respectively) (Figure 6C). Finally, HER-2 levels correlated with the urine protein:creatinine ratio ($P = 0.0227$) and with levels of other LN biomarkers, namely, MCP-1 ($P = 0.0304$) and VCAM-1 ($P < 0.0001$) (Figure 6D).

Regulation of MCP-1 and VCAM-1 levels by miRNAs. MCP-1 and VCAM-1 are thought to participate in the infiltration of the kidneys by inflammatory cells, a well-known process in LN pathogenesis. Our in silico studies identified VCAM-1 as a possible miR-30b target in LN kidneys (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). We therefore examined MCP-1 and VCAM-1 levels in mesangial cells overexpressing miR-26a and miR-30b, and we found significantly decreased expression of both MCP-1 and VCAM-1 in cells overexpressing miR-26a and decreased expression of VCAM-1 in cells overexpressing miR-30b (see Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract). These data suggest that proliferation and inflammation are both influenced by these miRNAs.

DISCUSSION

Our data support a model in which type I IFNs, via the transcription factor IRF-1, induce the expression of HER-2. This pathway down-regulates miR-26a and miR-30b, thereby driving cell proliferation through de-repression of genes involved in the cell cycle (see Supplementary Figure 6, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39219/abstract).

Our initial discovery study began with pediatric SLE patients. The pediatric age group was selected because LN is particularly prevalent and severe in children with SLE (2,19), and comorbidities such as diabetes mellitus or hypertension are not as frequent. We found a kidney miRNA signature specific for LN, characterized by a significant decrease in miR-26a, miR-30b, and miR-4286. Manipulation of these miRNAs in human mesangial cells showed that they participate in cell cycle regulation by controlling the expression of several key genes, including *CCNE2* and *E2F8*, which are involved in the G_1/S transition (20,21). *CCNE2* had also been predicted to be a target of both miR-26a and miR-30b by the miRanda algorithm.

In LN there is a large spectrum of morphologic changes in the glomerular, tubulointerstitial, and vascular compartments of the kidneys, but cell proliferation is central to LN pathogenesis. Not only is mesangial hypercellularity one of the first manifestations of the disease, but also the presence of crescents is a well-known negative prognostic factor.

Decreased miR-26a expression has also been identified in several malignancies (18,22–39). Consistently, miR-26a overexpression inhibits cell proliferation (25,28,29,40), usually through the control of *CCNE2* expression (22,25,40). Dysregulation of miR-26a has also been associated with other autoimmune diseases, such as rheumatoid arthritis (41) and idiopathic pulmonary fibrosis (42).

Similarly, miR-30b is associated with cell proliferation (30,43). Down-regulation of miR-26a and miR-30b has also been reported in the sera of patients with scleroderma and those with systemic sclerosis (44), which are fibrotic diseases that affect multiple tissues. In our study, significantly decreased miR-26a and miR-30b levels were found in LN, a disease characterized precisely by cell proliferation and fibrosis, the 2 processes found to be associated with these miRNAs in other diseases.

The stability of miRNAs in urine and the ease of obtaining such samples make these molecules promising candidates as noninvasive biomarkers. We showed that levels of miR-26a and miR-30b are significantly decreased not only in the kidneys of LN patients, but also in the urine of these patients. These data should be further validated with new robust strategies to quantify urinary miRNAs using standardized internal controls.

Trastuzumab targets the HER-2 extracellular domain and blocks its downstream pathways by inhibiting the dimerization of HER-2 and by promoting the internalization and cleavage of HER-2 molecules. Currently, trastuzumab is used for the treatment of patients with HER-2–positive breast and gastric cancer, where it causes arrest at the G_1 phase by up-regulating miR-26a and miR-30b (18). The mechanism by which HER-2 regulates miR-26a and miR-30b is not known. Our studies showed a dramatic increase of HER-2 expression in the kidneys of NZM2410 mice and LN patients. Furthermore, mesangial cells exposed to trastuzumab had elevated levels of miR-26a and miR-30b and down-regulation of genes associated with mitosis and cell proliferation, demonstrating that this pathway is central to the regulation of miR-

26a and miR-30b. Whether this pathway could be exploited therapeutically is an intriguing question.

Other members of the epidermal growth factor (EGF) family have been shown to participate in the pathogenesis of renal diseases. In a mouse model of crescentic glomerulonephritis, the activation of EGF receptor (EGFR) in podocytes resulted in development or progression of the disease, while the pharmacologic blockage or genetic deletion of one of its ligands improved the course of the disease and prevented the infiltration of inflammatory cells (45). Moreover, activation of EGFR in cultured podocytes led to proliferation, dedifferentiation, and migration, processes that are thought to occur in crescent formation in vivo (45). However, we did not find HER-2 overexpression in other types of glomerulonephritides also characterized by mesangioproliferation, such as IgA nephropathy, poststreptococcal glomerulonephritis, and granulomatosis with polyangiitis. We therefore hypothesized that lupus-associated factors drive HER-2 overexpression. The effects of IFNα and IRF-1 in human mesangial cells were evaluated, since the role of these 2 factors in SLE etiopathogenesis has been previously identified (46–48). We showed that both IFNα and IRF-1 increased HER-2 expression in human mesangial cells and that IRF-1 was also associated with a significant decrease of miR-30b. The ChIP-Seq data available on the University of California, Santa Cruz Genome Bioinformatics platform (http://genome.ucsc.edu/) are consistent with the binding of IRF-1 to the HER-2 promoter region in K562 cells, which further supports our hypothesis that IRF-1 controls HER-2 expression.

HER-2 in the urine was significantly increased in LN patients and it was associated with proliferative disease, as expected from its known role in cell growth. Urinary HER-2 levels were also significantly correlated with the urine protein:creatinine ratio as well as with levels of MCP-1 and VCAM-1, which are recognized LN biomarkers (49–51). HER-2 levels likely capture a distinct pathologic mechanism in LN, and while the association between biomarkers is statistically robust, there are clear differences between inflammatory mediator detection and HER-2 detection, which will require additional investigation.

This study identified a novel pathway and potential biomarkers, using multiple confirmatory strategies. Nevertheless, there are some limitations. While our initial miRNA analysis and pathologic studies relied on pediatric cases of LN, the urine analyses were performed in adults, where achieving an adequate sample size was possible. This precluded direct analysis of the association of expression of HER-2 with expression of miRNA from the same patient. In addition, the majority of patients were African American. New data should be obtained in different age groups and in different racial backgrounds in order to further validate our findings. Larger, longitudinal studies will be needed. Nevertheless, our data highlight a novel, previously unexpected pathway and provide a substantial foundation for further investigation of these potential biomarkers. For decades, proliferation has been recognized as the hallmark of LN, and the identification of this pathway opens the door to novel therapeutic interventions.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Costa-Reis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Costa-Reis, Russo, Sullivan.

Acquisition of data. Costa-Reis, Russo, Colonna, Maurer, Gallucci, Schulz, Kiani, Petri.

Analysis and interpretation of data. Costa-Reis, Russo, Zhang, Sullivan.

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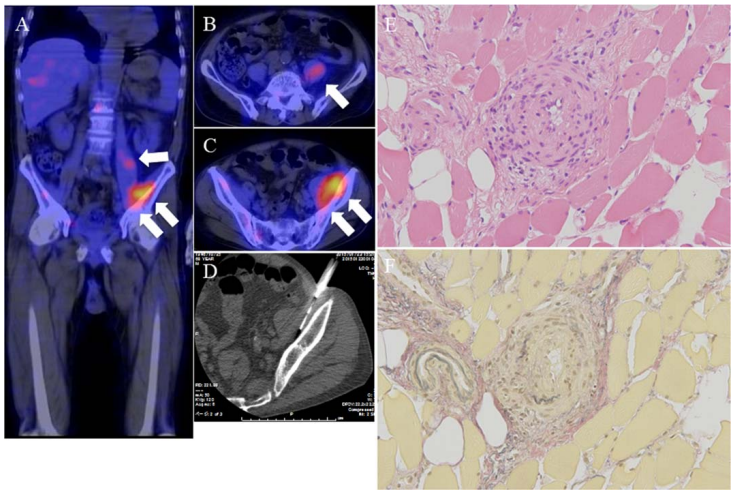
Clinical Images: Polyarteritis nodosa of the iliopsoas muscle



The patient, a 67-year-old man, presented with a 2-week history of fever, malaise, and low back pain. There had been no response to antibiotic treatment. The biochemical profile showed elevated levels of C-reactive protein (CRP) (143 mg/liter; normal <3) and ferritin (39,924 ng/ml; normal 39.4–340); testing for antineutrophil cytoplasmic antibodies yielded negative results. Blood cultures were negative, and hepatic and renal function were normal. Notably, gallium scintigraphy with computed tomography (CT) revealed a swollen left iliopsoas muscle with high gallium uptake (**A–C**) (**arrows**). Enhanced CT showed no visceral aneurysms. CT-guided needle biopsy of the iliopsoas muscle (**D**) demonstrated large collections of mononuclear inflammatory cells in the walls of a medium-sized muscular artery, as also seen on histologic assessment with hematoxylin and eosin staining (**E**). Elastic–van Gieson staining showed fragmentation in the internal elastic lamina in the same artery (**F**). The patient was diagnosed as having muscular polyarteritis nodosa confined to the left iliopsoas muscle. Treatment with prednisolone was initiated, and high-dose intravenous immunoglobulin therapy was later added. After treatment, his symptoms improved, and the serum CRP and ferritin levels normalized. The iliopsoas muscle edema with high gallium uptake completely resolved. He was discharged without further complications. Polyarteritis nodosa of a muscle is quite rare and has been reported to be restricted to the lower legs (1,2). To our knowledge, this is the first reported case of polyarteritis nodosa confined to the iliopsoas muscle. This case should remind clinicians to consider single-organ vasculitis as a rare cause of fever of unknown origin or isolated lesions found on imaging.

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Appendix A.4 – Paper ***Uma Nova Era no Diagnóstico e Tratamento da Síndrome Hemofagocítica,*** published in *Acta Pediátrica Portuguesa* in December 2016.

ARTIGO DE REVISÃO / REVIEW ARTICLE

Uma Nova Era no Diagnóstico e Tratamento da Síndrome Hemofagocítica

A New Era for Diagnosis and Treatment of Haemophagocytic Syndromes

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Resumo

A síndrome hemofagocítica caracteriza-se pela ocorrência de inflamação generalizada associada à hiperativação de macrófagos e linfócitos T e hipersecreção de citocinas. Pode ser primária, quando causada por defeitos genéticos na citotoxicidade, ou secundária a infeções, neoplasias ou doenças reumatológicas. O quadro clínico típico inclui febre, hepatoesplenomegalia, linfadenopatias, citopenias, disfunção do sistema nervoso central e falência multiorgânica. O diagnóstico precoce é difícil, mas crucial para o início rápido do tratamento e melhoria do prognóstico. Novos critérios clínicos foram desenvolvidos recentemente para o diagnóstico de síndrome hemofagocítica em diferentes contextos. Dados da ciência básica, sobretudo relativos a modelos animais, têm contribuído para um melhor entendimento dos mecanismos de doença e têm permitido fundamentar o uso de fármacos biológicos nas formas secundárias. Neste artigo serão explorados os avanços recentes ao nível do diagnóstico e tratamento da síndrome hemofagocítica e será feita uma revisão dos vários modelos da sua patogénese.

Palavras-chave: Criança; Inflamação; Linfohistiocitose Hemofagocítica/diagnóstico; Linfohistiocitose Hemofagocítica/tratamento

Abstract

Haemophagocytic syndromes are characterized by overwhelming inflammation related to uncontrolled activation of macrophages and T-lymphocytes and hypersecretion of cytokines. These syndromes can be primary, when caused by genetic defects in cytotoxicity, or secondary to rheumatic diseases, infection, or malignancy. Usually patients are acutely ill with fever, splenomegaly, lymphoadenopathies, cytopaenia, central nervous system dysfunction and progressive multiorgan failure. Diagnosis is challenging, but essential to provide prompt treatment and increase survival. Recently new criteria have been developed to diagnose haemophagocytic syndromes in different settings. Data from mouse models have helped to better understand the pathogenesis of these conditions and established strong foundations for the use of biological therapies in secondary forms of haemophagocytic syndromes. In this review we highlight recent advances in the diagnosis and treatment of haemophagocytic syndromes and focus on current models of the pathogenesis of these conditions.

Keywords: Child; Inflammation; Lymphohistiocytosis, Hemophagocytic/diagnosis; Lymphohistiocytosis, Hemophagocytic/therapy

Introdução

A síndrome hemofagocítica caracteriza-se por inflamação generalizada, associada à secreção exagerada de citocinas pró-inflamatórias e ativação descontrolada de macrófagos e linfócitos T.[1-3] A presença na medula óssea de hemofagócitos, macrófagos ativados que fagocitam outras células hematopoiéticas, dá o nome à síndrome.

A síndrome hemofagocítica pode ser primária, também conhecida como linfohistiocitose hemafogocítica familiar, um grupo de doenças genéticas raras causadas por alterações da citotoxicidade; ou secundária a infeções, neoplasias ou doenças reumatológicas. O termo "síndrome de ativação macrofágica" é muitas vezes utilizado para nomear o último grupo.

O diagnóstico é difícil, particularmente em contexto de cuidados intensivos, assemelhando-se à síndrome de resposta inflamatória ou à falência multiorgânica.[4] A sua identificação é crucial, uma vez que a estratégia terapêutica passa frequentemente pelo recurso a imunossupressores, abordagem diferente da utilizada na maioria das situações de falência multiorgânica. O início precoce da terapêutica é determinante no prognóstico.[5]

Manifestações clínicas

As crianças com síndrome hemofagocítica encontram-se, na maioria dos casos, gravemente doentes. É frequente o início súbito de febre alta, citopenias, disfunção hepática, sintomas neurológicos e falência multiorgânica.[5-7]

Em doentes com artrite idiopática juvenil sistémica (AIJS), a febre alta intermitente, típica de doença ativa, altera-se para um padrão contínuo de febre com o início da síndrome de ativação macrofágica.[7]

As manifestações mucocutâneas podem ser variadas, incluindo exantema, eritrodermia, edema, petéquias, púrpura, equimoses e hemorragia das mucosas.[5-9]

O atingimento do sistema nervoso central ocorre frequentemente, podendo manifestar-se por cefaleia, letargia, irritabilidade, desorientação, ataxia, neuropatia periférica sensitiva e motora, convulsões e coma.[5-7] Por vezes, estas alterações dominam o quadro clínico e são anteriores ao aparecimento de outros sintomas.[10-13]

Mais recentemente, foi descrito envolvimento pulmonar nas formas secundárias, representando um fator de mau prognóstico.[14] Outras manifestações possíveis incluem serosite, miocardite, cardiomegalia, arritmias e falência cardíaca.[5,7,15]

O envolvimento renal também pode ocorrer, com progressão para insuficiência renal e falência multiorgânica.[5,7] Nestes casos, a mortalidade é elevada.[16]

No exame objetivo, os sinais mais frequentes são hepatoesplenomegalia e linfadenopatias generalizadas.[5-7,9,17]

Alterações laboratoriais

As alterações laboratoriais mais frequentemente observadas nestes doentes incluem pancitopenia e ferritina elevada.[5,6,9,15]

Numa grande coorte de doentes com síndrome hemofagocítica níveis de ferritina superiores a 500, 5 000 e 10 000 ng/mL foram observados em 93%, 42% e 25% dos doentes, respetivamente.[18] Um outro estudo mostrou que um nível de ferritina superior a 10 000 ng/mL tem uma sensibilidade de 90% e uma especificidade de 96% para o diagnóstico de síndrome hemofagocítica, permitindo fazer o diagnóstico diferencial com sépsis e insuficiência hepática.[19] A ferritinemia é importante não só para o diagnóstico, mas também como marcador da atividade da doença, resposta à terapêutica e prognóstico.[6,9]

É também frequente detetar-se aumento das enzimas hepáticas,[20,21] da bilirrubina e gamaglutamil transferase,[6,9,11,22] da lactato desidrogenase[21] e dos triglicéridos.[23]

Estes doentes apresentam habitualmente um perfil de coagulação alterado, com tempo de protrombina e tromboplastina parcial ativada prolongados, hipofibrinogenemia e presença de produtos de degradação de fibrina.[6,7,9,15]

Hiponatremia e hipoalbuminemia também são alterações frequentes.[6,9,15]

Uma diminuição da velocidade de sedimentação eritrocitária é comum e deve levantar a suspeita de síndrome hemofagocítica, sobretudo em doentes com AIJS, que usualmente apresentam uma velocidade de sedimentação aumentada.[6,9,15] O quociente ferritina/velocidade de sedimentação superior a 80 tem uma alta sensibilidade e especificidade na diferenciação entre AIJS e síndrome de ativação macrofágica.[24]

Novos marcadores para síndrome hemofagocítica têm sido recentemente descritos. O receptor solúvel alfa da interleucina (IL)-2 (sIL2Rα/sCD25) está muito aumentado na fase aguda da síndrome de ativação macrofágica quando comparado com AIJS de início recente.[25] O CD163, recetor da haptoglobina e marcador específico de macrófagos, encontra-se igualmente muito aumentado no soro, medula óssea e baço de doentes com síndrome hemofagocítica.[25,26] O CD163 e o CD25 solúvel (sCD25) correlacionam-se com a atividade da doença, refletindo o grau de ativação dos macrófagos e dos linfócitos T respetivamente.[25]

Exame anatomopatológico

A presença de hemofagocitose no aspirado ou biópsia de medula óssea pode ser útil, mas não é suficiente ou necessária para o diagnóstico de síndrome hemofagocítica. Destaca-se que a hemofagocitose nem sempre é demonstrável na fase inicial da doença e doentes com AIJS podem ter hemofagocitose sem evidência clínica de síndrome de ativação macrofágica.[15,27,28] O exame anatomopatológico pode ser, assim, considerado quando o diagnóstico é duvidoso, mas não é essencial. O diagnóstico e tratamento nunca devem ser adiados pela ausência de hemofagocitose na medula óssea

Diagnóstico diferencial

A síndrome hemofagocítica pode ser desencadeada por múltiplos estímulos, incluindo infeção, neoplasia ou doença reumatológica. Pode ocorrer também no contexto de defeitos genéticos na citotoxicidade. Neste caso, os sintomas surgem geralmente nos primeiros anos de vida, com uma sobrevivência mediana inferior a dois meses na ausência de tratamento.[29]

No contexto de doenças reumatológicas, a síndrome de ativação macrofágica é particularmente comum em doentes com AIJS, ocorrendo em pelo menos 7-13% dos doentes[7,15,17] e podendo ser subclínica ou assumir uma forma mais ligeira em outros 30-40%.[15-25] Também pode ocorrer em doentes com lúpus eritematoso sistémico,[30] doença de Sjogren,[31] dermatomiosite,[31-33] esclerose sistémica, poliarterite nodosa, doença de Kawasaki, artrite reumatoide,[31] artrite relacionada com entesite,[17,31] sarcoidose[31] e em casos de síndromes de

febre periódica.[17,34,35] Pode surgir espontaneamente ou ser desencadeada por uma infeção ou pelo efeito tóxico de um fármaco.[5,17]

As neoplasias, sobretudo leucemias e linfomas, também se associam a síndrome hemofagocítica, existindo, frequentemente uma infeção concomitante.[36-38] A síndrome hemofagocítica pode preceder, ocorrer simultaneamente à identificação da neoplasia[39] ou ser desencadeada pela imunossupressão induzida pela quimioterapia.[36] A probabilidade de uma doença maligna subjacente aumenta com a idade.[36]

Infeções virais, particularmente pelo vírus Epstein-Barr, podem desencadear formas primárias bem como secundárias de síndrome hemofagocítica.[7,33,40,41] Outros estímulos infeciosos conhecidos incluem citomegalovírus,[41] parvovírus B19,[7,33,42] vírus herpes simplex,[43,44] varicella-zoster,[7] vírus herpes humano-8,[45,46] coxsackie,[7] influenza,[47,48] parainfluenza,[49] parechovirus[50] e vírus de imunodeficiência humana (VIH).[51]

Apesar de menos comum, as síndromes hemofagocíticas podem ocorrer igualmente no contexto de infeções bacterianas por *Staphylococcus aureus*,[41] *Escherichia coli*,[41] *Salmonella enteritidis*,[7] *Mycoplasma pneumoniae*,[52] *Ehrlichia chaffeensis*,[53] *Brucella mellitensis*[41] e *Mycobacterium tuberculosis*[41]; parasitas, incluindo *Leshmania spp41* e *Plasmodium falciparum*[54] e *Plasmodium vivax*[55]; e fungos,[33] como *Histoplasma capsulatum*[41,56] e *Coccidiodes*.[57]

A síndrome hemofagocítica também pode ocorrer no curso de histiocitose de células de Langerhans,[33] síndrome de Chédiak-Higashi,[58] síndrome de Griscelli tipo 2[59] e síndrome de Hermanski-Pudlak tipo 2.[60] Imunodeficiências adquiridas, incluindo VIH, transplante de células hematopoiéticas[61] e transplante de fígado/rim[62-64] também se associam a maior risco para síndrome hemofagocítica.

Doentes com intolerância à proteína lisinúrica,[65] imunodeficiência combinada grave,[66] síndrome de DiGeorge,[66] síndrome de Omenn´s[66,67] e doença granulomatosa crónica,[66] podem apresentar-se de uma forma semelhante a doentes com síndrome hemofagocítica primária. Nestes casos, as infeções são o estímulo mais frequente.[66]

Existem várias doenças que se podem assemelhar a síndrome hemofagocítica, entre as quais a síndrome linfoproliferativa autoimune, a púrpura trombótica trombocitopénica, a síndrome hemolítica urémica, a microangiopatia trombótica, doenças hepáticas primárias e a reação a drogas com eosinofilia e sintomas sistémicos.

Critérios de diagnóstico

O diagnóstico de síndrome hemofagocítica primária é feito de acordo com os critérios propostos pela *Histiocyte Society* em 2004,[68] que incluem febre, esplenomegalia, bicitopenia, hipertrigliceridemia e/ou hipofibrinogenemia, hiperferritinemia, sIL2Rα/sCD25 elevado, atividade de células NK diminuída ou ausente e evidência de hemofagocitose na medula óssea, baço ou gânglios linfáticos. Do total de oito critérios, um mínimo de cinco devem ser preenchidos, a menos que a história familiar ou molecular seja consistente com linfohistiocitose hemofagocítica familiar.[68] Estes critérios não estão validados, no entanto, para o diagnóstico de síndrome hemofagocítica secundária.

Em 2009, a *Pediatric Rheumatology European Society* propôs os seguintes critérios para síndrome de ativação macrofágica em doentes com lúpus eritematoso sistémico[69]:

- Critérios clínicos
  - Febre (> 38ºC)
  - Hepatomegalia (≥ 3 cm abaixo do rebordo costal)
  - Esplenomegalia (≥ 3 cm abaixo do rebordo costal)
  - Manifestações hemorrágicas (púrpura, equimoses fáceis ou hemorragia das mucosas)
  - Disfunção do sistema nervoso central (irritabilidade, desorientação, letargia, cefaleia, convulsão ou coma)
- Critérios laboratoriais
  - Citopenia afetando duas ou mais linhagens (hemoglobina ≤ 9,0 g/dL; leucócitos ≤ 4,0 x 10^9/L ou plaquetas ≤ 150 x 10^9/L)
  - Aspartato aminotransferase (AST) > 40 U/L
  - Desidrogenase láctica (LDH) > 567 U/L
  - Fibrinogénio ≤ 1,5 g/L
  - Triglicéridos > 178 mg/dL
  - Ferritina > 500 µg/L
- Critérios histopatológicos
  - Evidência de hemofagocitose no aspirado de medula óssea

O diagnóstico de síndrome de ativação macrofágica requer a presença simultânea de pelo menos um critério clínico e dois critérios laboratoriais.[69] O aspirado de medula óssea deve ser considerado apenas se o diagnóstico for duvidoso.[69]

É particularmente difícil distinguir entre síndrome de ativação macrofágica e agudização de AIJS, dado que têm uma apresentação clínica semelhante. Os critérios da *International League of Associations for Rheumatology* para o diagnóstico de AIJS incluem febre, hepatoesplenomegalia e linfadenopatia, achados partilhados pela síndrome de ativação macrofágica.[70] Doentes com esta patologia também apresentam níveis elevados de ferritina,[71] D-dímeros[72] e tempo de protrombina[73] e até 53% podem apresentar hemofagocitose no aspirado da medula óssea.[15] Pensa-se que a AIJS e a síndrome de ativação macrofágica sejam uma entidade única, em que a síndrome de ativação macrofágica é a forma mais grave do espetro.[15]

Recentemente, novos critérios clínicos e laboratoriais foram estabelecidos para o diagnóstico de síndrome de ativação macrofágica em doentes com AIJS que se encontrem febris[74]:

- Ferritina > 684 ng/mL
- E pelo menos dois dos seguintes:
    - Plaquetas ≤ 181 x 10^9/L
    - AST > 48 U/L
    - Triglicéridos > 156 mg/dL
    - Fibrinogénio ≤ 360 mg/dL

Curiosamente, os critérios não incluem manifestações clínicas, com exceção da febre, o que reflete a noção de que os sintomas são frequentemente tardios e semelhantes aos de outras doenças. Alterações laboratoriais mesmo que subtis e ainda dentro da normalidade devem, por isso, levantar a suspeita de síndrome de ativação macrofágica.[74]

Abordagem clínica

A anamnese deve incluir questões sobre infeções recentes, neoplasias, imunodeficiências, doenças reumatológicas, fármacos e história familiar de sintomas semelhantes. No exame objectivo deve ser pesquisada a existência de hepatoesplenomegalia, linfoadenopatias, exantema, hemorragias cutâneo-mucosas e alterações neurológicas.

Da avaliação laboratorial e imagiológica, deve fazer parte ecografia abdominal,[75] hemograma, estudo da coagulação, fibrinogénio, AST, alanina aminotransferase (ALT), albumina, perfil lipídico e ferritina.[75]

Se nenhum estímulo for identificado, devem ser procuradas doenças infecciosas, com radiografia de tórax, hemo e urocultura, esfregaço sanguíneo, serologias para vírus Epstein-Barr, citomegalovírus, herpes simplex, *varicella-zoster*, parvovirus, influenza, adenovirus, hepatite B, hepatite C e VIH.[75] Na suspeita de neoplasia, citometria de fluxo periférica, estudos de imagem e biópsia da medula óssea são exames apropriados.[75] Recomenda-se o rastreio da deficiência de perforina por citometria de fluxo.[76] Quando alterado, devem ser estudadas mutações nos genes *PRF1, UNC13D, STX11, STXBP2* e *RAB27A*.[75]

Tratamento

Em doentes clinicamente estáveis e com um estímulo identificado, é recomendado o início do tratamento dirigido à condição desencadeante. Em caso de infeção deve ser, assim, iniciada imediatamente terapêutica dirigida ao microrganismo suspeito. Em casos de infeção a vírus Epstein-Barr está recomendado rituximabe, um anticorpo monoclonal anti CD20, 375 mg/m^2 semanalmente, durante uma a quatro semanas.

Relativamente à linfohistiocitose hemofagocítica familiar o tratamento mais utilizado é o recomendado pela *Histiocyte Society* (Protocolo HLH-94). Inclui uma fase de indução de oito semanas com dexametasona e etoposido,[11] e, na presença de sintomas neurológicos, metotrexato intratecal. Esta estratégia terapêutica revelou-se eficaz no aumento da sobrevida,[77] mas apenas o transplante de células hematopoiéticas pode garantir a cura.[78,79] Em 2004, o protocolo foi sujeito a uma revisão (HLH-2004), tendo sido incluída a ciclosporina A na fase de indução. No entanto, a indução com terapêutica tripla associa-se a neurotoxicidade, pelo que as recomendações atuais favorecem uma fase de indução com etoposido e dexametasona, seguida de manutenção com ciclosporina A.[80]

Em 2015, foi realizado o primeiro estudo prospetivo em adultos com síndrome hemofagocítica, desencadeada maioritariamente por neoplasia ou infeção. O etoposido e dexametasona foram usados na fase de indução e, nos casos de ausência de remissão parcial em duas semanas, foi implementado tratamento com doxurrubicina, etoposido e metilprednisolona. Os resultados foram bastante promissores, ocorrendo uma resposta ao tratamento em 76% dos doentes.[81]

Relativamente a doentes com síndrome de ativação macrofágica, recomenda-se que o tratamento inicial seja efetuado com corticosteroides isoladamente, sem recurso a etoposido. A monoterapia com corticoide leva a remissão em 71% dos doentes.[7,82] Quando a resposta não é favorável, outros agentes imunossupressores devem ser utilizados, em vez dos protocolos HLH-94 ou HLH-2004.[75]

A ciclosporina A bloqueia a ativação de linfócitos T, reduz a produção de citocinas inflamatórias e inibe a expressão de moléculas coestimuladoras na superfície de células dendríticas.[83-85] É eficaz no tratamento da síndrome hemofagocítica primária e em casos de síndrome de ativação macrofágica graves ou cortico-resistentes.[5,7,17,86-88] Em alguns doentes ocorre apirexia e melhoria dos valores laboratoriais nas primeiras 12 a 24 horas após o início da terapêutica com ciclosporina A.[89] Estes dados levaram alguns autores a propôr a utilização de ciclosporina A, 2-5 mg/kg/dia, como tratamento de primeira linha na síndrome de ativação macrofágica.[89,90]

Os primeiros fármacos biológicos usados para tratar a síndrome de ativação macrofágica foram os inibidores do fator de necrose tumoral α.[91-94] Atualmente deixaram de estar recomendados, uma vez que foram identificados vários doentes em que a síndrome de ativação macrofágica poderá ter sido desencadeada pelo uso destes fármacos.[95-98]

O bloqueio da interleucina (IL)-1 e da IL-6 parecem ser mais promissores. Anakinra, um antagonista do receptor da IL-1, é altamente eficaz no tratamento da AIJS.[99] Existem também múltiplas descrições de doentes com AIJS e síndrome de ativação macrofágica que melhoraram quando foi iniciada a terapêutica com anakinra.[100-104] Em contraste, existem também alguns casos de doentes com AIJS em que o desencadear da síndrome de ativação macrofágica se associou ao tratamento com anakinra (1-2 mg/kg/dia).[99,105] Neste grupo de doentes a causa exata foi difícil de estabelecer e a suspensão do trata-

mento não foi necessária, tendo-se verificado inclusivamente melhoria do quadro quando utilizadas doses superiores. Anakinra continua, assim, a ser uma terapêutica recomendada para a síndrome de ativação macrofágica refratária.[2]

O canakinumab, um anticorpo monoclonal que bloqueia a IL-1β, é também eficaz no tratamento da AIJS (4 mg/kg)[106] e, em doses mais elevadas, no tratamento da síndrome de ativação macrofágica (7,5-12,5 mg/kg).[107]

O tocilizimab, um anticorpo monoclonal anti-IL-6, é usado com bons resultados em doentes com AIJS.[108,109] O seu papel no controlo da síndrome de ativação macrofágica ainda não está bem definido, sobretudo porque existem vários casos descritos de doentes com AIJS cuja síndrome de ativação macrofágica foi diretamente relacionada com a utilização de tocilizumab.[110-112]

Recentemente, foram descritos 30 doentes que desenvolveram síndrome hemofagocítica após receberem terapias biológicas, tendo-se concluído que as infeções induzidas pelos agentes biológicos eram os estímulos mais prováveis para a síndrome hemofagocítica e não o fármaco em si.[41]

Em conclusão, corticosteroides e ciclosporina A são os agentes mais frequentemente utilizados para o tratamento da síndrome de ativação macrofágica. Recomendam-se pulsos de metilprednisolona intravenosa 30 mg/kg em três dias consecutivos, seguidos de 2-3 mg/kg/dia em duas a quatro tomas diárias. Se a resposta aos corticoides não for imediata, a administração parentérica de ciclosporina A (2-5 mg/kg/dia) deve ser iniciada.[113] Em doentes refratários a este tratamento, existe uma evidência crescente que terapias biológicas, particularmente inibidores de IL-1, podem ser úteis.[2]

O etoposido é reservado para casos de linfohistiocitose hemofagocítica familiar, síndromes hemofagocíticas secundárias a neoplasias ou infeções, ou em doentes gravemente doentes refratários às restantes terapêuticas.[88,104]

Em doentes com linfohistiocitose hemofagocítica familiar, neoplasias hematológicas, sintomas recorrentes e/ou atingimento do sistema nervoso central está recomendado o transplante de células hematopoiéticas. O prognóstico é melhor se o doente estiver em remissão no momento do transplante.[18,114]

Terapêutica pré-transplante com alemtuzumab (anticorpo monoclonal anti CD52), fludarabina e melfalano está associada a menor risco de toxicidade e maior probabilidade de sobrevivência.[115,116]

Novas modalidades terapêuticas estão atualmente a ser desenvolvidas, incluindo terapia génica para a correção de defeitos da perforina e a utilização de um anticorpo monoclonal humano anti-interferão γ para o tratamento das formas primárias.[117,118] Em dois modelos animais diferentes de formas primárias e secundárias de síndrome hemofagocítica, o tratamento com ruxolitinib, um inibidor das Janus kinases (JAK) 1 e 2, levou a uma melhoria franca do quadro clínico, pelo que estes fármacos são considerados muito promissores.[119]

Prognóstico

Doentes com linfohistiocitose hemofagocítica familiar têm um tempo médio de sobrevivência de aproximadamente dois meses após o diagnóstico, se não tratados.[29,77] No caso dos doentes submetidos ao protocolo HLH-94 a taxa de sobrevivência é de 54% aos 6,2 anos,[18,114] sendo inferior nas crianças diagnosticadas antes dos 6 meses e nos doentes com atingimento neurológico.[18,114] De 124 doentes submetidos a transplante de células estaminais hematopoiéticas alogénico, a sobrevivência aos cinco anos foi de 66%, sendo melhor no subgrupo de doentes em remissão na altura do transplante.[18,114]

As taxas de sobrevivência são maiores na síndrome de ativação macrofágica. Um estudo recente indica uma taxa de mortalidade de 8% em doentes com AIJS que desenvolveram esta complicação.[5]

Valores muito elevados de ferritina e ausência de diminuição significativa destes níveis com o tratamento são factores de mau prognóstico em todos os tipos de síndrome hemofagocítica.[120]

Podem verificar-se recorrências da síndrome, sendo mais provável no primeiro ano após a doença aguda inicial. O risco de recorrência pode ser minimizado reduzindo a exposição a fatores desencadeantes. Dado que recorrências após vacinação têm sido relatadas, é recomendado evitar vacinas nos primeiros seis meses e devem ser administradas uma de cada vez.[121]

Novos dados sobre a patogénese da síndrome hemofagocítica

Em 1998, descobriu-se que doentes com linfohistiocitose hemofagocítica familiar apresentavam defeitos ao nível da função das células NK.[122] No ano seguinte foi descrita, pela primeira vez, a associação a mutações do gene que codifica a proteína perforina.[123] A perforina localiza-se nos grânulos citotóxicos de células NK e linfócitos T e é secretada mediante conjugação com as células alvo. Na presença de cálcio, insere-se na membrana das células alvo, polimerizando para formar um poro. A granzima B, uma protease também encontrada nas células citotóxicas, entra nas células alvo pelos poros produzidos pela perforina, induzindo apoptose. Dados provenientes de modelos animais indicam que a perforina é importante na defesa contra cancro e agentes infeciosos intracelulares.[124,125]

Tem sido igualmente sugerido que a perforina controla a proliferação linfocitária.[123] A deficiência de perforina pode contribuir deste modo para a ativação linfocitária persistente e, consequentemente, para a produção de citocinas pro-inflamatórias, incluindo o interferão Ɣ que ativa os macrófa-

gos. Estima-se que 15-40% dos doentes com linfohistiocitose hemofagocítica familiar tenham mutações no gene que codifica a perforina.[123]

Mutações no gene *UNC13D*, o qual codifica a proteína *MUNC13-4*, têm sido implicadas em 10-30% dos doentes com linfohistiocitose hemofagocítica familiar.[126] Esta proteína participa na ancoragem e fusão de grânulos citotóxicos com a membrana citoplasmática.

Mutações nos genes *STX11* (Syntaxin 11) e *STXBP2* (Syntaxin Binding Protein 2) codificadores de proteínas igualmente importantes no tráfego de grânulos citotóxicos, também têm sido associadas ao desenvolvimento de formas primárias de síndrome hemofagocítica.[127-129]

Em conclusão, desde a descrição do papel da perforina, diversas proteínas relacionadas com a produção, exocitose e função de grânulos citotóxicos de linfócitos T CD8+ e células NK têm sido encontradas alteradas em doentes com linfohistiocitose hemofagocítica familiar.[130] Esta lista vai certamente aumentar nos próximos anos com novos dados a surgirem de estudos de sequenciação genómica/exómica.

Curiosamente, nos últimos anos a distinção entre síndromes hemofagocíticas primárias e secundárias está a tornar-se mais difícil à medida que novas causas genéticas são identificadas. Num estudo de 2014, em 281 doentes italianos com síndrome hemofagocítica classificados como formas não familiares, 43 (15%) tinham mutações monoalélicas em um dos genes associados a linfohistiocitose hemofagocítica familiar.[131] Doentes com formas secundárias podem apresentar, assim, mutações monoalélicas nos mesmos genes que produzem linfohistiocitose hemofagocítica familiar quando ambos estão mutados.[131] Conceptualmente, estes dados são consistentes com um modelo *multi-hit*: indivíduos geneticamente suscetíveis quando expostos a determinados estímulos desenvolvem hiper-inflamação.[26] Todos estes dados permitem concluir que a função citotóxica não é apenas importante como função efetora imune, mas também para resolução da inflamação.[26]

Modelos animais permitiram compreender com mais exatidão a patogénese da síndrome hemofagocítica. Ratinhos com deficiência de perforina (Prf-/-) infetados com vírus linfocítico de coriomeningite exibem características clínicas semelhantes às de doentes com linfohistiocitose hemofagocítica familiar.[132] Este modelo permitiu compreender a importância do interferão γ na patogénese das formas primárias de doença. Destaca-se que ratinhos *Prf*-/- que também apresentam deficiência de interferão γ encontram-se protegidos da ocorrência de anemia[132] e a injeção de interferão γ em ratinhos sem deficiência da perforina é suficiente para causar hemofagocitose e anemia, de forma dose-dependente.[132] Ratinhos transgénicos que seletivamente exprimem um recetor do interferão γ mutado dominante-negativo nas células da linhagem macrofágica não desenvolvem hemofagocitose e estão protegidos de anemia quando injetados com interferão γ.[132] Estes dados sugerem, assim, que o interferão γ deve atuar diretamente nos macrófagos para a ocorrência de hemofagocitose *in vivo*.

De acordo com o modelo atual da síndrome hemofagocítica primária, os linfócitos T citotóxicos e células NK falham na sua função de morte de células infetadas, causando a persistência do antigénio. Adicionalmente, células citotóxicas falham no envio de sinais apoptóticos apropriados para a remoção de linfócitos T ativados e macrófagos, causando expansão persistente destas células e secreção aumentada de citocinas pro-inflamatórias, incluindo interferão γ, TNF α, IL-6, Il-10, Il-12, Il-16 e IL-18.[15,133-135] Como resultado da estimulação contínua por estas citocinas, principalmente interferão γ, os macrófagos tornam-se hemofagócitos.[2,136]

Outro modelo animal utiliza ratinhos portadores do transgene I|6, os quais produzem altos níveis de IL-6.[137] Estes ratinhos quando estimulados com ligandos de receptores *Toll-like* (TLR) apresentam uma mortalidade aumentada, o que permite especular sobre uma possível causa de síndrome de ativação macrofágica no contexto de AIJS. Níveis circulantes altos de IL-6, típicos desta doença, podem predispor a uma hiper-resposta a infeções.[137] Além disso, um estudo recente demonstrou que, quer em ratinhos quer em humanos, a IL-6 contra-regula a atividade citotóxica de células NK, reduzindo os níveis de perforina e granzima B, na ausência de alteração da exocitose de grânulos.[138] Este é uma nova e interessante conexão entre as síndromes hemofagocíticas primárias e secundárias.

Outro modelo murino de síndrome de ativação macrofágica baseia-se na estimulação repetida do TRL-9.[139] Este modelo é importante dado que não é dependente de defeitos genéticos ou de uma infeção viral, mimetizando melhor o que ocorre no ser humano. O TRL-9 é um receptor para ácido desoxirribonucleico (ADN) contendo sequências CpG não metiladas. Existem múltiplos estudos que associam a hiperativação do TRL-9 à ocorrência de síndrome de ativação macrofágica. Nos doentes com AIJS existe uma assinatura genética consistente com sinalização crónica de TLR/IL-1β[140] e o vírus Epstein-Barr, um dos estímulos mais frequentes de síndromes hemofagocíticas secundárias, é um vírus de ADN que ativa o TLR-9.[141] O modelo murino de estimulação TRL-9 replica, por isso, o ambiente que permite que a síndrome de ativação macrofágica ocorra em hospedeiros geneticamente predispostos. Ratinhos submetidos a estimulação de TLR-9 desenvolvem características similares à síndrome de ativação macrofágica.[139] A doença neste modelo é fulminante, se existir um bloqueio concomitante de IL-10.[139] Curiosamente, polimorfismos de IL10 associados a menor função estão associados a AIJS, mostrando que a IL-10 pode desempenhar um papel protetor.[142] Ao contrário do que é observado noutros modelos animais, experiências de depleção revelaram que células T, células NK ou células B não são determinantes no fenótipo. Adicionalmente, quadros fulminantes ocorrem na ausência de interferão γ, IL-12, TNF-α,

INF-α e INF-β, demonstrando que esta condição se deve a uma rede complexa de citocinas e é independente de qualquer citocina isoladamente. [143]

Outra contribuição importante deste modelo foi a dissociação entre anemia e hemofagocitose. Ratinhos tratados com estimulação repetida do TLR-9 exibem anemia na ausência de hemofagocitose, enquanto ratinhos interferão Ɣ-/- com bloqueio de IL-10 apresentavam hemofagocitose sem anemia.[139,143] Estes dados sugerem que é a diseritropoiese mediada por interferão Ɣ, e não a hemofagocitose, o mecanismo dominante da anemia no contexto da síndrome de ativação macrofágica.[143]

Ainda que no passado a presença de hemofagocitose tenha sido associada à ocorrência de anemia e à atividade da doença, não existe demonstração de causalidade. Pelo contrário, estudos recentes sugerem que os hemofagócitos podem ter uma função de regulação da inflamação. A eritrofagocitose pode providenciar um substrato para a produção de heme oxigenase-1, a qual limita a resposta inflamatória.[15,144] Estudos em modelo animal e em humanos apoiam esta hipótese.[33] Os hemofagócitos capturados no baço de ratinhos com bloqueio de IL-10 e estimulação repetida do TLR-9 exibem um aumento da expressão de genes relacionados com o fenótipo M2 dos macrófagos, uma característica indicativa de função anti-inflamatória.[33] Destaca-se ainda que o CD 163, um marcador do fenótipo M2 dos macrófagos, é altamente expresso em hemofagócitos humanos.[145]

Curiosamente, foi descoberto que os hemofagócitos são uma fonte importante de IL-10, uma citocina importante para o controlo da resposta inflamatória, e que quando são incapazes de a produzir, a mortalidade induzida por vírus aumenta.[146] Os hemofagócitos podem constituir, por isso, uma máquina de *feedback* negativo com funções reguladoras em vez de serem os causadores da doença.[33,146]

Conclusões

A síndrome hemofagocítica pode ser desencadeada por múltiplos estímulos, mas, independentemente da sua causa, é caracterizada por um estado de híper-inflamação. Dados da ciência básica conduziram a um modelo da patogénese da síndrome hemofagocítica no qual indivíduos geneticamente suscetíveis desenvolvem inflamação não controlada quando expostos a determinados estímulos. Isto verifica-se não apenas nos doentes com linfohistiocitose hemofagocítica familiar, os quais apresentam defeitos inatos na citotoxicidade, e desenvolvem a síndrome hemofagocítica após um desencadeante infeccioso, mas também nas formas secundárias. Em doentes com AIJS, por exemplo, a exposição contínua a IL-6 está associada a uma hiper-resposta a estímulos infecciosos.

Outra manifestação comum em todas estas síndromes é a hemofagocitose. Curiosamente, assistiu-se a uma mudança radical na forma como interpretamos o papel dos hemofagócitos: não só não parecem necessários para a ocorrência de anemia nestas condições, como podem mesmo desempenhar uma função reguladora anti-inflamatória. Consequentemente, poderá ser necessária uma nova nomenclatura para estas síndromes, dado que a hemofagocitose já não é considerada o passo mais importante na sua patogénese.

Nos últimos anos assistiu-se igualmente a uma mudança profunda na abordagem clínica das síndromes hemofagocíticas, em que novos critérios de diagnóstico foram estabelecidos e novas estratégias terapêuticas foram testadas. Vivemos, assim, numa era de grandes progressos que certamente terão impacto na sobrevivência destes doentes.

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Appendix A.5 – Paper ***Alternative Activation of Laser-Captured Murine Hemophagocytes***, published in *Arthritis and Rheumatology*, June 2014.

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BRIEF REPORT

Alternative Activation of Laser-Captured Murine Hemophagocytes

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Objective. **Hemophagocytes (HPCs) are activated macrophages that have engulfed other hematopoietic cells. Although HPCs are rarely identified in normal spleen tissue and bone marrow, an excess of these macrophages characterizes many cytokine storm syndromes, particularly macrophage activation syndrome and hemophagocytic lymphohistiocytosis. This study was undertaken to assess the functions of HPCs and their significance in acute inflammatory conditions.**

Methods. **HPCs were generated in wild-type mice using repeated stimulation with Toll-like receptor 9 (TLR-9) and interleukin-10 receptor blockade. RNA was extracted from HPCs that had been isolated by laser-captured microdissection. Transcriptional profiles of the HPCs were then compared to those of resting splenic macrophages. In addition, bone marrow samples were obtained from a diverse cohort of patients in whom excess hemophagocytosis was identified by clinical bone marrow biopsy or aspiration. The bone marrow samples were analyzed by immunohistochemistry for markers of classic (CD64) or alternative (CD163 and CD206) macrophage activation.**

Results. **Differential gene expression and gene set enrichment analyses of murine HPCs identified upregulation of genes and gene sets associated with alternative activation of HPCs. Immunohistochemical analyses of HPCs in human bone marrow samples showed universal staining of HPCs for CD163, but rarely for CD206 or CD64.**

Conclusion. **Laser-captured murine TLR-9–induced HPCs had a transcriptional profile similar to that of alternatively activated macrophages. In addition, HPC expression of CD163 was confirmed in a uniquely diverse cohort of patients with hemophagocytic syndromes. Collectively, these data support the hypothesis that HPCs have both immunoregulatory and clean-up functions.**

Macrophages reside in organs throughout the body, where they are involved in diverse functions, including pathogen sensing, pro- and antiinflammatory immune responses, and wound healing. Macrophage activation has been described in a continuum from classically activated (M1) to alternatively activated (broadly categorized as M2) (1). M1 macrophages have proinflammatory functions that often result in tissue damage, while M2 macrophages participate in immunoregulation and tissue remodeling. Thus, macrophage functions can be fluid and varied, with either proinflammatory or antiinflammatory actions, depending on the mix of signals being received.

Morphologically, hemophagocytes (HPCs) are macrophages that have engulfed other hematopoietic cells. Detection of hemophagocytosis is an important aspect in the diagnosis and management of macrophage activation syndrome (MAS) and hemophagocytic lymphohistiocytosis (HLH). MAS and HLH are complex cytokine storm disorders that can complicate various infectious, rheumatic, or malignant diseases (2). HLH can also be caused by primary genetic defects in cytotoxicity.

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Studies in animals and humans have suggested that HPCs have both pro- and antiinflammatory roles. Evidence for a proinflammatory role derives from the important effects of interferon-γ (IFNγ) on macrophages, whereby IFNγ acts to drive disease progression in animal models of MAS and HLH (3), and from the localization of proinflammatory cytokines in liver biopsy specimens from patients with MAS (4). The evidence pertaining to alternative activation includes observations of the expression of a scavenger receptor, CD163, on HPCs (2), as well as detection of antiinflammatory functions in murine erythrophagocytes, identified by flow cytometry (3,5).

In this study, we show that the transcriptional program of morphologically identified murine HPCs is consistent with a profile of alternative activation. In addition, we confirm the expression of CD163 on human bone marrow HPCs from a uniquely broad cohort of patients with hemophagocytic syndromes. While the roles of macrophages in hemophagocytic syndromes remain imprecise, these results suggest that murine Toll-like receptor 9 (TLR-9)–induced HPCs are alternatively activated and that their presence may be beneficial to the control or clearance of inflammation.

MATERIALS AND METHODS

Isolation of murine HPCs. Fulminant MAS was induced in mice using repeated stimulation with TLR-9 (via CpG administration) and interleukin-10 receptor (IL-10R) blockade, as previously described (6). Splenic touch preparations (touch preps) were made on nuclease-free polyethylene naphthalate membrane–coated slides (Zeiss), followed immediately by staining of the touch preps with Wright-Giemsa stain. A pediatric hematopathologist (MEP) with expertise in hemophagocytic syndromes morphologically identified HPCs from TLR-9–stimulated, IL-10R–blocked mice or resting macrophages from saline-treated mice. From each mouse, 20 cells were captured using a Zeiss PALM (photoactivated localization microscopy) laser microdissection system (Figures 1A and B). Thereafter, the cells were isolated, pooled, and processed in aggregate.

RNA isolation and microarray. Complementary DNA (cDNA) libraries were generated from the RNA of pooled, microdissected cells using a WT-Ovation One-Direct Amplification System (NuGEN) according to the manufacturer's instructions. Fragmented cDNA was then hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST Arrays, and then washed, stained, and scanned with an Affymetrix GeneChip Scanner 3000 7G system.

Transcriptional analysis. Affymetrix Expression Console software was used to perform quality control, excluding 2 chips from each group and leaving 4 biologic replicates per group for analysis. Microarrays were preprocessed using robust multiarray analysis (Gene Expression Omnibus accession no. GSE47430) (7). Probe sets lacking gene symbol annotation or with a mean $\log_2$ intensity of <5 among the HPC samples were filtered out. Log_2-transformed expression data were analyzed using R statistical computing language. Differentially expressed genes (DEGs) were defined as those with at least 1.5-fold difference in expression in HPCs as compared to resting macrophages. DEGs were tested for statistical significance using Student's *t*-tests, with correction for multiple testing using the false discovery rate (FDR). Functional enrichment analysis among DEGs was performed using the DAVID database (version 6.7) (8). Gene Ontology terms for biologic pathways, molecular function, and cellular components were tested, as were Kyoto Encyclopedia of Genes and Genomes (KEGG) biologic pathways. Genes that passed the filtering criteria were used as the background gene list in the enrichment analysis.

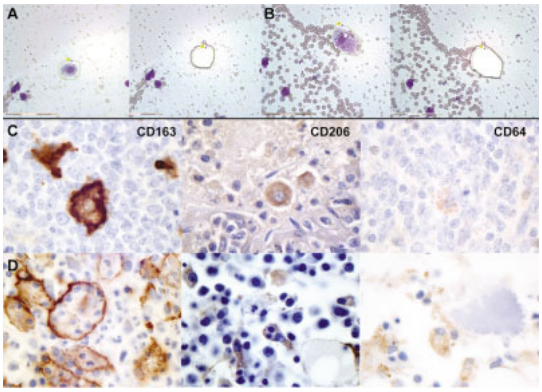

Figure 1. Evaluation of the transcriptional profile of murine and human hemophagocytes (HPCs). **A** and **B,** Resting murine splenic macrophages (**A**) and murine HPCs (**B**) were isolated by laser-captured microdissection and assessed for activation status. **C** and **D,** HPCs in bone marrow biopsy specimens from patients with hemophagocytic disease were immunostained for CD163, CD206, or CD64 (**D**). As positive controls for the respective immunohistochemical stains, tonsil tissue from patients without hemophagocytic disease was assessed (**C**). Original magnification × 100; counterstaining with hematoxylin.

Gene set enrichment analysis (GSEA). Gene sets were identified by searching the Molecular Signatures database for the term "inflammation" (9). Gene sets with substantial overlap were excluded. Initially, 17 gene sets were included in this analysis, but the Phagocytosis gene set was excluded because too few probes were included in our data set (Table 1). MOgene expression data were translated into Human Genome Organisation (HUGO) gene symbols before comparison with selected gene sets, as per GSEA protocol. Gene sets were analyzed using gene set permutation, and prior to analysis, we chose the following threshold for significant enrichment: a nominal *P* value of ≤ 0.05 and FDR of $\leq 5\%$ (9).

Patient selection and immunohistochemical analysis. We performed an unbiased, retrospective search of the pathology database of the Children's Hospital of Philadelphia be-

Table 1. Gene set enrichment analysis of TLR-9–induced hemophagocytes compared to resting macrophages*

Gene set	Size	N-ES	Nominal *P*	FDR
Proteasome†	43	2.42	<0.001	<0.001
Up-regulated in M2 vs. M1†	73	1.85	<0.001	0.009
NOD-like receptor signaling†	54	1.74	0.002	0.022
Innate immunity signaling	97	1.49	0.015	0.133
Actin regulation	195	1.48	0.007	0.116
Cytosolic DNA sensing	46	1.48	0.058	0.101
TLR signaling	90	1.41	0.044	0.127
Up-regulated in M1 vs. M2	76	1.32	0.080	0.188
Endocytosis	160	1.23	0.12	0.283
TLR-3 cascade	56	1.04	0.391	0.545
TLR-4 cascade	25	0.93	0.556	0.717
IL-10 pathway	17	0.84	0.663	0.839
Integrin pathway	37	0.61	0.963	1
TLR-9 cascade	20	0.54	0.969	0.989
Inflammatory pathway	25	−0.95	0.547	1
IL-1R pathway	30	−0.67	0.921	0.946

* TLR-9 = Toll-like receptor 9; N-ES = normalized enrichment score; FDR = false discovery rate; IL-10 = interleukin-10; IL-1R = IL-1 receptor.
† Significantly enriched gene set, as defined in Materials and Methods.

tween 1998 and 2011, for bone marrow specimens obtained from pediatric patients whose clinical report documented a history of excessive hemophagocytosis. Thirty-seven samples from 34 patients were identified and verified as having excess hemophagocytosis. These samples had been decalcified, fixed in acetic acid–zinc–formalin (AZF), and paraffin-embedded, according to the institution's protocol.

Markers of classic macrophage activation (CD64) (1:25, clone 10.1; Abcam) or alternative macrophage activation (CD163 [1:200, clone 10D6; Vector Laboratories] and CD206 [1:500, clone 5C11; Abnova/Novus Biologicals]) were detected in the bone marrow samples by immunohistochemistry. Heat antigen retrieval was utilized for CD64 and CD163 (at pH 6 and pH 9, respectively). Avidin–biotin complex signal amplification was then used for CD206, and a polymeric-based method was used for CD64 and CD163. Detection was performed with horseradish peroxidase and 3,3′-diaminobenzidine chromogen, and the sections were counterstained with hematoxylin. AZF-fixed tonsils from patients without hemophagocytic disease were used as controls (Figure 1C) for the assessment of adequate positive or negative staining. All bone marrow samples from patients with hemophagocytic syndromes were evaluated for the presence or absence of immunostained HPCs (Figure 1D), performed in a blinded manner by a hematopathologist (MEP) with expertise in hemophagocytic diseases.

Chart review identified the specific clinical information related to each sample (details available from the corresponding author upon request). This study was approved by the Institutional Review Board of the Children's Hospital of Philadelphia.

RESULTS

Up-regulation of genes associated with macrophage activation and regulation of inflammation in TLR-9–induced murine HPCs. Forty-five genes met our criteria for differential expression (Table 2 and results not shown). No genes with decreased expression in HPCs as compared to resting macrophages were found to be statistically significantly down-regulated. Genes encoding the ribosomal protein subunits Rps20 and Rpl35a were among the most highly up-regulated, suggesting that HPCs may regulate global protein synthesis. Consistent with this notion, functional enrichment analysis of all DEGs suggested that "translation" was a significant biologic function of HPCs (Benjamini-corrected $P = 0.002$ for this Gene Ontology biologic process annotation). We further found that the KEGG "Ribosome" pathway was significantly enriched (Benjamini-corrected $P = 1.6 \times 10^{-7}$).

Another group of up-regulated genes comprised those related to cellular energetics. Two genes encoding

Table 2. Highly differentially expressed genes in Toll-like receptor 9–induced hemophagocytes compared to resting macrophages*

Gene	Protein product	Fold change	*P*	FDR
Rps20	Ribosomal protein S20	22.6	0.0063	0.146
β2m	β_2-microglobulin	12.5	0.0292	0.206
Rpl35a†	Ribosomal protein L35A	8.5	4.29×10^{-4}	0.055
Saa3	Serum amyloid A3	7.9	0.0014	0.055
Ifitm2	Interferon-inducible transmembrane protein 3	6.7	0.0445	0.210
Cox6c	Cytochrome c oxidase, subunit Vic	6.2	0.0238	0.174
Ftl†	Ferritin light chain	5.8	0.0028	0.055
Tmsb10	Thymosin β10	4.9	0.0430	0.210
GAPDH†	Glyceraldehyde 3 phosphate dehydrogenase	4.9	0.0019	0.055
Cox6a1	Cytochrome c oxidase subunit VIa, polypeptide 1	4.2	0.0388	0.210
Usmg5	Up-regulated during skeletal muscle growth 5	4.2	0.0412	0.210
Tmsb4x	Thymosin β4	4.1	0.0427	0.210
S100a6	S100 calcium binding protein A6 (calcyclin)	4.1	6.27×10^{-4}	0.055

* Genes associated with ≥4-fold induction in hemophagocytes as compared to resting macrophages. FDR = false discovery rate.
† Target identified by more than one probe.

subunits of the mitochondrial respiratory protein complex cytochrome c oxidase (Cox6c, Cox6a1) were up-regulated, as was the gene for the glycolytic enzyme GAPDH. Accordingly, the KEGG "Oxidative Phosphorylation" pathway was significantly enriched in genes differentially expressed by HPCs (Benjamini-corrected $P = 4.2 \times 10^{-4}$). Thus, these data suggest that TLR-9–induced HPCs are acting to meet the demands for energy and protein synthesis.

Genes associated with macrophage stimulation and activation were also among the most differentially expressed in murine HPCs. The β_2-microglobulin (B2m) and interferon-inducible transmembrane protein 3 (Ifitm2) genes, both of which are associated with antiviral responses, were up-regulated, as were the genes for serum amyloid A3 (Saa3) and S100 calcium binding protein A6 (S100a6), both of which have a role in the acute-phase response. Up-regulation of the ferritin light chain (Ftl) gene by HPCs is consistent with the presence of hyperferritinemia, a characteristic of MAS/HLH. Finally, the homologs thymosin β10 (Tmsb10) and β4 (Tmsb4) may play regenerative and antiinflammatory roles in macrophages.

GSEA findings of alternative polarization of murine TLR-9–induced HPCs. To refine our analysis of differential gene expression, we tested for up-regulation of gene sets associated with a variety of relevant transcriptional programs in HPCs. Only 3 gene sets fulfilled our criteria for significance: genes involved in proteasomal degradation, NOD-like receptor signaling genes, and the set of genes up-regulated by M2-polarized macrophages as compared to M1-polarized macrophages (10) (see Table 1 and results available from the corresponding author upon request). Notably, the set of genes up-regulated in M1 macrophages as compared to M2 macrophages was not significantly enriched in HPCs.

Uniform expression of CD163, but only rare expression of CD206 or CD64, in HPCs from a diverse human cohort. We identified a longitudinal cohort of patients whose bone marrow samples by biopsy or aspiration showed excess hemophagocytosis, regardless of the diagnosis. These samples represented hematologic malignancies (lymphoma [n = 1], Langerhans' cell histiocytosis [n = 3], and posttransplant lymphoproliferative disease [n = 3]), infections (Epstein-Barr virus [n = 6], bacterial [n = 2], parvovirus [n = 1], and fungal virus [n = 1]), immunodeficiencies (paroxysmal nocturnal hemoglobinuria [n = 1] and Munc13-4 deficiency [n = 1]), rheumatic diseases (systemic and other forms of juvenile idiopathic arthritis [n = 9] and dermatomyositis [n = 1]), and idiopathic causes of hemophagocytosis (n = 9).

Regardless of the origin of disease or treatment stage, the bone marrow samples demonstrated positive staining of HPCs for CD163 in all tested samples (Figure 1D). CD163 is a scavenger receptor that binds and internalizes hemoglobin–haptoglobin complexes, which then activate heme oxygenase 1 and induce the synthesis of ferritin (11). Five samples, representing diverse diseases, stained positively for CD206 (Figure 1D), a mannose receptor and marker of alternative activation or M2 macrophage activation (1). Two samples, both from patients with systemic infections, showed positive staining of HPCs for CD64, the Fcγ receptor type I and a marker of classic/M1 macrophage activation (Figure 1D) (1). No samples demonstrated positive staining of HPCs for both CD64 and CD206.

DISCUSSION

Hemophagocytes are enigmatic cells that appear in a variety of inflammatory contexts. Their presence as part of the diagnostic criteria for MAS and HLH often influences important medical decisions regarding evaluation and treatment. However, our poor understanding of the function of these cells complicates interpretation of their presence or absence. Our analysis of the transcriptional program of morphologically identified murine TLR-9–induced HPCs suggests that they are alternatively activated.

The fulminant TLR-9–induced MAS model, which includes IL-10R blockade, was chosen on the basis of the homogeneity of hemophagocytosis, the absence of confounding by infection or genetic alteration, and the fact that mice treated with TLR-9 stimulation alone or IL-10R blockade alone failed to develop HPCs (6). Examination of the genes most up-regulated by HPCs showed induction of Ftl and Saa3, among other genes. Astoundingly high ferritin levels are characteristic of MAS/HLH, and ferritin has been associated with antiinflammatory transcripts in systemic juvenile idiopathic arthritis (12). Production of both ferritin and serum amyloid A3 has been associated with antiinflammatory activity in macrophages (11,13).

We also found that cytochrome oxidase genes, oxidative phosphorylation pathways, and the canonically glycolytic enzyme GAPDH were up-regulated in HPCs. Although this might suggest that both glycolysis and oxidative phosphorylation are induced, GAPDH is increasingly associated with a variety of nonglycolytic

functions, including inhibition of cytokine translation (14).

The M2 versus M1 gene set was generated by evaluating genes that were up-regulated by in vivo–skewed M2 macrophages as compared to M1 macrophages (10). We found that this M2-associated gene set, but not the M1 gene set, was strongly associated with genes differentially up-regulated by HPCs (Table 1), thus supporting the notion that HPCs have a transcriptional profile of alternative activation. The other enriched gene sets, comprising those associated with NOD-like receptor signaling and proteasome activity, may be associated with scavenger receptor activity (15) or the degrading requirements of hemophagocytosis, respectively.

RNA from laser-captured splenic macrophages served as the basis for our microarrays. Notably, macrophages derived from splenic touch preps may not be entirely comparable to those found in the bone marrow. Moreover, our gene expression analyses were limited by the necessary pooling of RNA from individual cells from any single mouse, and by small input amounts of RNA. Thus, the potential for M1 versus M2 heterogeneity exists within mice and even within individual cells.

Furthermore, these results have not been validated by quantitation of specific targets. The limited starting material made protein quantitation impossible. In addition, the process of amplifying small-input RNA limited our ability to quantitate at the RNA level: the RNA fragments that result from this process, while of ideal size for microarray, were of insufficient length for conventional quantitative polymerase chain reaction. However, our analyses were able to detect changes associated with macrophage activation. Specifically, our transcriptomic data suggested that the dominant program of murine HPCs induced by TLR-9 stimulation and IL-10R blockade is skewed toward alternative activation. Accordingly, recent data support the notion that TLR-9–driven HPCs arise independent of a number of proinflammatory cytokines, including IFNγ (6), and may exert antiinflammatory effects (5). Given the prominent role of IL-10 in alternative macrophage activation (1), future investigations may help illuminate the specific role of IL-10 in the development of HPCs.

Our immunohistochemical analyses for selected markers in human bone marrow samples identified CD163 expression by HPCs in a cohort that, to our knowledge, is unique in terms of its breadth of diagnoses. The association of CD163 with alternative activation is well established (11). Future studies should attempt to further validate the findings from TLR-9–stimulated murine HPCs through the evaluation of human samples.

In this study of morphologically identified HPCs, we have shown transcriptional evidence for alternative activation of murine HPCs from an infection-free model, as well as immunohistochemical evidence that human HPCs from diverse sources express CD163. These data support the body of literature suggesting that HPCs are alternatively activated macrophages that occur as a common response to systemic inflammation. In MAS/HLH, it is likely that there is heterogeneity in macrophage function, and therapeutic depletion of all macrophages could actually be detrimental. Further study is warranted to better understand the induction of hemophagocytosis, the precise functions of HPCs, and the roles that these cells play in regulating the immunopathologic processes in hemophagocytic syndromes.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Canna had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Canna, Costa-Reis, Bernal, Paessler, Behrens.

Acquisition of data. Canna, Costa-Reis, Bernal, Chu, Paessler.

Analysis and interpretation of data. Canna, Costa-Reis, Bernal, Sullivan, Paessler, Behrens.

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Errata

DOI 10.1002/art.38706

In Table 2 of the article by Freed et al in the December 2011 issue of *Arthritis & Rheumatism* (pages 3733–3739), the numbers of rheumatoid arthritis patients and control subjects positive for the resistance epitope D^{70} were shown incorrectly. The correct numbers of rheumatoid arthritis patients and control subjects, respectively, were 209 and 299.

DOI 10.1002/art.38693

In the article by Kanno et al in the February 2013 issue of *Arthritis & Rheumatism* (pages 492–502), the concentration of α2-antiplasmin (α2AP) used in experiments was shown incorrectly in several figures and/or figure legends. In Figures 1C and E, Figures 2A and B, Figures 4A and B, and Figures 5D and E, the α2AP concentration should have been shown as 2 n*M*.

DOI 10.1002/art.38707

In the lower panel of Figure 1 of the article by Hanly et al in the November 2013 issue of *Arthritis & Rheumatism* (pages 2887–2897), the data points for intractable headaches and cluster headaches at the enrollment visit were inadvertently reversed. The correct values are 6.49% for intractable headaches at the enrollment visit and 2.36% for cluster headaches at the enrollment visit. Additionally, in the text of the article the prevalence of headache at 10 years was incorrectly stated. The first full sentence on page 2888 should have read, "The prevalence of headache increased to 42% after 10 years" and the last sentence on page 2890 (continuing onto page 2891) should have read, "The established proportion of patients who ever reported a headache (Kaplan-Meier estimate) increased to 42% after 10 years (Figure 2)."

DOI 10.1002/art.38708

In the article by Golding et al published in the November 2013 issue of *Arthritis & Rheumatism* (pages 2898–2906), acknowledgment of an author's funding was inadvertently omitted. The paragraph on funding support in the footnotes on the first page of the article should have included the statement, "Dr. Golding's work was supported by a VA Career Development Award (IK2 CX-000649)."

DOI 10.1002/art.38709

The byline of the Reply letter by Golding et al published in the May 2014 issue of *Arthritis & Rheumatology* (pages 1403–1404) contained several errors. First, the correct spelling of the second author's name is Ethan M. Shevach, MD. Second, the correct name of the institute where Dr. Hasni is employed is the National Institute of Arthritis and Musculoskeletal and Skin Diseases. Third, Dr. Illei is currently employed by MedImmune, Gaithersburg, MD.

We regret the errors.

B. PAPERS AS CO – AUTHOR

Appendix B.1 – Paper ***New Insights into the immunopathogenesis of Systemic Lupus Erythematosus***, published in *Nature Reviews Rheumatology*, November 2016.

New insights into the immunopathogenesis of systemic lupus erythematosus

George C. Tsokos[1], Mindy S. Lo[2], Patricia Costa Reis[3] and Kathleen E. Sullivan[4]

Abstract | The aetiology of systemic lupus erythematosus (SLE) is multifactorial, and includes contributions from the environment, stochastic factors, and genetic susceptibility. Great gains have been made in understanding SLE through the use of genetic variant identification, mouse models, gene expression studies, and epigenetic analyses. Collectively, these studies support the concept that defective clearance of immune complexes and biological waste (such as apoptotic cells), neutrophil extracellular traps, nucleic acid sensing, lymphocyte signalling, and interferon production pathways are all central to loss of tolerance and tissue damage. Increased understanding of the pathogenesis of SLE is driving a renewed interest in targeted therapy, and researchers are now on the verge of developing targeted immunotherapy directed at treating either specific organ system involvement or specific subsets of patients with SLE. Accordingly, this Review places these insights within the context of our current understanding of the pathogenesis of SLE and highlights pathways that are ripe for therapeutic targeting.

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Progress in understanding systemic lupus erythematosus (SLE) has been hampered by disease heterogeneity. Patients with SLE can present with diverse organ involvement as well as diverse autoantibodies. In fact, SLE probably represents several heterogeneous diseases that fall into a broad clinical phenotype of systemic autoimmunity. Many patients with SLE have mild disease, whereas others have a catastrophic presentation and life-threatening progression. Our current understanding of the factors that drive the different phenotypes in SLE is limited; however, in spite of an imperfect understanding of the pathogenesis of SLE, great progress has been made over the past 50 years and mortality is now only 10% within 10 years (compared with 50% within 3 years in the 1960s)[1]. Nevertheless, infections related to immune suppression, cardiovascular disease, and renal failure constitute a substantial burden, and medical costs and costs related to lost productivity are high[2].

The pathogenesis of SLE hinges on loss of tolerance and sustained autoantibody production (FIG. 1). Unlike self-limited autoantibody processes, such as autoimmune haemolytic anaemia, SLE is generally a lifelong condition. One of the key concepts in pathogenesis is an imbalance between apoptotic cell production and disposal of apoptotic material (FIG. 2). Nuclear antigens are typically not accessible to the immune system, but during the course of apoptosis the cell membrane forms blebs that pinch off from the cell and contain fragmented cellular material, including nuclear antigens[3]. Such apoptotic debris is normally cleared rapidly and would not be accessible to the immune system. In humans, approximately 1 billion neutrophils undergo apoptosis every day and increases in the apoptotic cell load can be generated by exposure to ultraviolet light, infections, and toxins, which are all known to be associated with SLE. Persistent apoptotic debris containing nucleic acids can stimulate an inflammatory response through the activation of nucleic acid recognition receptors, such as members of the Toll-like receptor (TLR) family[4]. Circulating apoptotic microparticles also prime neutrophils for extrusion of nuclear material, providing yet more antigen[5]. Nucleic acid recognition receptors control endogenous retroviruses, recognize viral pathogens, and defend against intracellular bacteria, and are strongly associated with type I interferon (IFN) production. Defects in these pathways are now strongly implicated in the pathogenesis of SLE, as both increasing disease susceptibility and directly causing monogenic forms of SLE (TABLES 1,2).

Type I IFNs and other cytokines promote B-cell differentiation and loss of tolerance. B cells can respond to nucleic acids through direct antigen recognition and via surface IgM receptors for proteins complexed with nucleic acids. Once autoantibodies have formed, B cells can also take up nucleic acids through Fc receptors and B-cell receptors recognizing Fc (rheumatoid factor)[6]. Once activated, these B cells mature, expand, and begin

Key points

- Our understanding of the pathogenesis of systemic lupus erythematosus (SLE) has changed rapidly over the past decade
- Refinements in our understanding over the past 3 years have led to the potential for precision targeting of therapeutic strategies
- Advances in epigenetic therapeutic agents and the manipulation of cells *ex vivo* have the potential to further improve patient care

to secrete more antibody, which enhances the adaptive immune response. T-cell and B-cell abnormalities have long been described in SLE and are thought to be central to the disease process. The autoantibodies identified in SLE are generally high-affinity, somatically mutated IgG, which suggests that they have arisen in germinal centres, where T cells provide help for class switching.

This framework for understanding SLE has been our model for the past ten years. It is founded on genetic data, *in vitro* analyses, and observations in mouse models. In this Review, we cover new insights that extend this model and offer the potential for novel therapeutic interventions in SLE. We discuss environmental and genetic factors that contribute to the risk of developing SLE, and examine how gene expression and regulation contributes to the phenotype of the disease and how these effects provide a window into the clinical features. Subsequent sections investigate mechanisms as we understand them from a cell biology perspective, and three examples of local tissue effects that can contribute to organ damage and modulate disease are also presented (FIG. 1).

Environmental risk factors

Imperfect disease concordance between monozygotic twins suggests that environmental factors influence the pathogenesis of SLE. Hormones and ultraviolet light have long been recognized as contributors to SLE[7,8]. Women comprise 90% of most SLE cohorts, and oestrogen and prolactin enhance immune responses through diverse mechanisms[9,10]. Ultraviolet light is thought to drive apoptosis, providing an immunologic stimulus. A possible connection between sunlight exposure and drug-induced lupus has also been identified. Ultraviolet light converts propranolol into a proinflammatory aryl hydrocarbon receptor ligand, possibly explaining its association with lupus-like disease[11].

Infections have been implicated in SLE for many years. Epstein–Barr virus and cytomegalovirus are considered to be SLE triggers[12], whereas *Helicobacter pylori*[13], hepatitis B virus[14], and parasite infections are thought to be protective[15]. One study found that herpes simplex virus type 2 transcripts were overexpressed in patients with systemic autoimmune diseases, although the role of immunosuppression in increased viral gene expression could not be eliminated[16]. Further data support a role for microorganisms in general. Lipopolysaccharide is a component of the cell wall of Gram-negative bacteria that can activate TLR4. Serum levels of lipopolysaccharide are increased in patients with SLE[17] and biomarkers of lipopolysaccharide engagement by TLR4, such as shedding of CD14, correlate with disease activity[18]. TLR4 activation promotes disease in mouse models of lupus[19]. Microbial stimulation of myeloid cells by TLRs is critical for antigen presentation to T cells[20]. These data suggest that chronic microbial translocation contributes to the pathogenesis of SLE. Bacterial biofilms represent another mechanism by which microorganisms interact with the immune system. Amyloid–DNA complexes, found in many biofilms, greatly increased the production of autoantibodies in lupus-prone mice[21]. At this point, the evidence seems clear that SLE is not uniformly caused by a single infection, but the role of bacteria and viruses generally in SLE represents an emerging area of study, and TLR antagonists are being evaluated as therapeutic agents.

The microbiome represents the collection of bacteria, viruses, and fungi that coexist on and in the human body. Collectively, microbial cells far outnumber human cells within the body and, while many were previously thought to be silent passengers, we now know that some can modulate the immune system[22]. Interest in the microbiome has grown exponentially, as it represents an attractive therapeutic target. In women with SLE, a lower *Firmicutes* to *Bacteroidetes* ratio was seen than in healthy individuals, even during times of remission[23]. In humans, microbiome studies are largely correlative, but mouse studies support a mechanistic role for the microbiome. Increased levels of *Bacteroidetes* were also seen in lupus-prone mice[24]. In a separate study, a manipulation that aimed to normalize the microbiome was beneficial in MRL/lpr mice[25]. The mechanism of the effect is not fully understood, but certain gut bacteria foster the development of regulatory T cells (T_{reg} cells)[26,27]. Developing the 'correct' (that is, healthy) microbiome might require neonatal exposure; one study found that development of antinuclear antibodies was dependent on bacterial colonization during the neonatal period in mice[28]. Although therapeutic alteration of the microbiome in humans has been limited to the setting of infections and inflammatory bowel disease, these studies represent an important proof of concept for the pursuit of additional studies in patients with SLE.

Genes and gene expression

One of the great advantages of pursuing genetic analyses in a highly heterogeneous disorder such as SLE is that it is otherwise difficult to understand which facets of disease represent susceptibility features, and which represent consequences of the disease.

Heritability of SLE and genetic studies

The heritability of SLE has long been recognized; a higher concordance rate in monozygotic twins than in dizygotic twins and the high sibling recurrence risk ratio support a strong heritability[29]. The major histocompatibility complex (MHC) was the first risk locus to be associated with SLE, and alleles within the MHC locus still confer the strongest genetic susceptibility for SLE in the general population today[30]. This seminal finding supports a disease process in which T cells play a central part, as their activation is dependent on MHC proteins (FIG. 2). In the past decade, numerous genome-wide

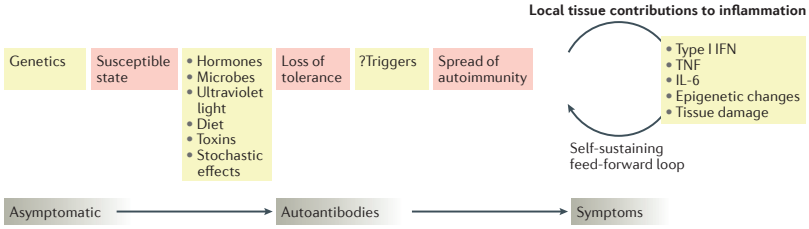

Figure 1 | **The current model of the pathogenesis of SLE.** The progression of systemic lupus erythematosus (SLE) can be divided into discrete stages. Environmental and genetic factors contribute to the development of disease. Triggers such as infection can elicit autoimmunity, but the elements that drive a sustained loss of tolerance and spreading of autoimmunity are poorly understood. Epigenetic changes, immune-complex deposition and autoantibody-mediated tissue damage can drive chronic inflammation and irreversible damage in end organs. IFN, interferon.

association studies (GWAS) have been performed and we now recognize over 40 loci that are confirmed to be associated with SLE[31]. Variants rarely lie in the coding exons, however, and most are instead thought to affect regulatory regions[32]. Regulatory risk variants may affect proximal genes or may act at a distance through chromosomal looping[33]; such variants might also have effects on multiple genes.

Genes associated with SLE are listed in TABLE 1. The overall genetic risks identified to date are limited, with each gene generally conferring a relative risk <2. The yet to be identified heritability could lie in rare variants that are individual to each kindred, or epigenetic effects. Most of the identified loci are associated with multiple autoimmune diseases[32,34]. The GWAS data have focused interest on three major cellular pathways, each influenced by many variants[35]: lymphocyte signalling, either within T cells or B cells; IFN signalling pathways involving either nucleic acid sensing or the production and response to IFNs; and clearance of immune complexes and other waste. Interestingly, a number of monogenic disorders are associated with an increased risk of developing SLE or a related phenotype (TABLE 2), and can be similarly categorized according to these same three pathophysiological pathways[36]. The key findings from these GWAS are identification of these three disease-associated pathways, variant sharing with other autoimmune diseases, and heterogeneity across populations and ethnic groups. Although GWAS have been criticized for failure to identify 'druggable' targets or major 'causative' variants, they have unquestionably moulded key concepts around the pathogenesis of SLE.

Epigenetic mechanisms in SLE

Genetics and genomics can be applied to evaluate heritable risks of disease. By contrast, epigenetics refers to the study of durable changes in gene expression that are not accompanied by alterations to the nucleotide sequence. Epigenetics is beginning to receive attention in the field of rheumatology. Epigenetic processes include DNA methylation, post-translational histone modifications, and noncoding RNAs that regulate gene expression.

DNA methylation was the first epigenetic change identified in patients with SLE. DNA methylation regulates gene expression by serving as a platform for repressive protein binding. Procainamide and other drugs known to induce lupus-like features are inhibitors of DNA methylation[37]. T cells from mice treated with these drugs are capable of inducing lupus in recipient mice[38]. These data not only clearly implicate the epigenome in SLE, but also highlight the central role of T cells. In humans, T cells from patients with active SLE have global DNA hypomethylation[39], especially those from patients with lupus nephritis[40]. The consequence of this hypomethylation is typically overexpression of genes, because DNA methylation is usually repressive. When examined on a genome-wide basis, IFN-stimulated genes (ISGs) were specifically hypomethylated in patients with SLE[40]. Further study revealed that, during a flare, naive $CD4^+$ T cells become primed for T_H2, T_H17, and T follicular helper (T_{FH}) cell immune responses through the activity of the chromatin-modifying enzyme histone–lysine *N*-methyltransferase EZH2 (REF. 41). Diet is known to influence DNA methylation, which may be one mechanism by which diet contributes to SLE susceptibility[42,43]. A further DNA modification is the formation of 5-hydroxymethylcytosine; levels of this modified form of cytosine are increased in T cells of patients with SLE and are also associated with increased gene expression[44].

Histones undergo a number of post-translational modifications that can serve as binding sites for proteins involved in regulation of gene expression. Histone modifications were initially studied in mouse models of lupus, in which treatment with histone deacetylase inhibitors improved disease features[45]. Furthermore, histone deacetylase 6 is overexpressed in MRL/lpr mice, and treatment directed at normalizing this enzyme improved the features of lupus[46,47]. The mechanisms by which these agents work is controversial, because these treatments seem to be highly immunosuppressive[48]. Nevertheless, these studies provide an important proof of principle that agents acting on epigenetic mechanisms could be useful in the treatment of human SLE. Our understanding of histone modifications in humans has been driven by two distinct approaches. In the first approach, total

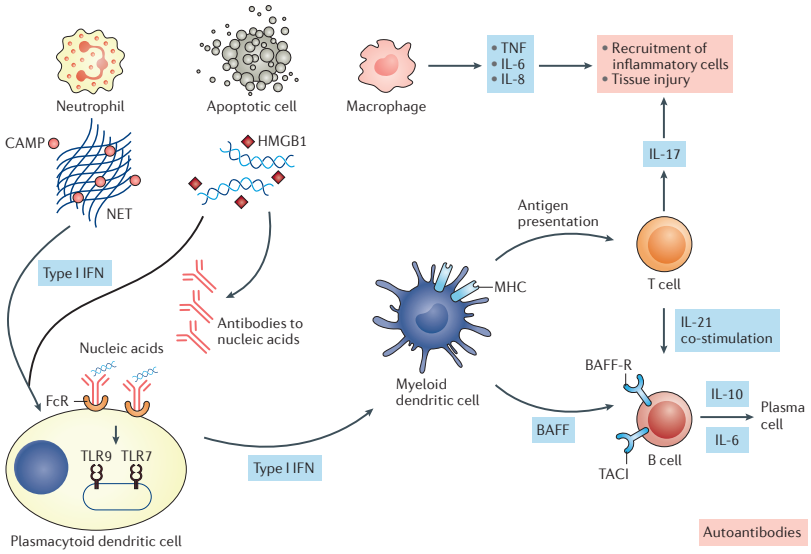

Figure 2 | **Cellular contributions to the development of SLE.** Neutrophils and apoptotic cells are at the apex of the cascade of pathogenetic mechanisms in systemic lupus erythematosus (SLE). They provide the critical ligands to drive expression of type I interferons (IFNs). Neutrophils represent a key inflammatory participant in organ damage; these cells also release neutrophil extracellular traps (NETs), a source of citrullinated peptide and nucleic acid antigens, via NETosis. Many cells produce type I IFNs, but plasmacytoid dendritic cells produce the highest levels of these cytokines. Apoptotic debris can also activate inflammatory cytokine expression which participates in the recruitment of cells into tissues. T cells and B cells both participate in autoreactivity, with B cells ultimately producing autoantibodies. T-cell production of IL-17 also contributes to organ infiltration by neutrophils. BAFF, B-cell activating factor; BAFF-R, BAFF receptor; CAMP, cathelicidin antimicrobial peptide; FcR, Fc receptor; MHC, major histocompatibility complex; TACI, transmembrane activator and cyclophilin ligand interactor; TLR, Toll-like receptor.

histone modifications were measured and shown to be aberrant in T cells from patients with SLE. These aberrations were corrected by treatment with mycophenolate mofetil[49]. The second approach utilized genome-wide analyses. In the initial analysis, histone H4 acetylation was shown to be globally increased in monocytes from patients with SLE[50]. This finding is consistent with those from DNA methylation studies[51] because both DNA hypomethylation and histone H4 hyperacetylation drive increased expression of target genes. Within the sites with increased H4 acetylation, potential binding sites for IFN regulatory factor 1 (IRF1) were identified, and IRF1 binding was directly shown to be increased in SLE[52]. IRF1 is a transcription factor downstream of type I IFN, which ties this finding of an altered epigenome back to the known influence of type I IFNs. Multiple histone modifications in enhancer regions were globally altered in SLE monocytes, which no doubt dictates altered cell behaviour[53]. Some histone modifications persist after stimulation, thereby 'bookmarking' genes for facilitated re-expression. This feature might contribute to disease chronicity[54,55]. One of the therapeutic efforts directed at the epigenome utilizes inhibitors of bromodomain-containing protein 4 (BRD4), a protein critical for enhancer function[56]. One such BRD4 inhibitor was demonstrated to be effective in a mouse model of lupus[57], again demonstrating the power of these genome-wide approaches to identify novel therapeutic targets.

MicroRNA regulation in SLE

MicroRNAs (miRNAs) target specific mRNAs for degradation and can regulate the abundance of multiple mRNAs[58]. Changes in miRNA expression have been identified in peripheral blood mononuclear cells and renal tissue from patients with SLE[59–61]. Plasma miRNAs can also be isolated and are presumed to be released from cells as a result of death, stress, or exocytosis[62]. Several of the miRNAs identified in patients with SLE seem to affect pathways that are central to the disease processes of SLE[61], such as TLR signalling and expression of ISGs[63]. Expression of miRNAs is very tissue-specific, and studies of miRNAs in kidney and peripheral blood samples from patients with SLE, have found none in either tissue[59,60]. Other data from human studies have implicated miR-30a in B cells, where this miRNA was thought to regulate expression of LYN, a critical signalling molecule[64]. In MRL/lpr mice, overexpression of miR-21 and miR-148a is responsible for the reduction in levels of

Table 1 | **GWAS-identified SLE susceptibility genes**

Pathway(s)	Loci implicated in SLE and other autoimmune diseases	Loci implicated only in SLE
Lymphocyte activation	*PTPN22*, *TNFSF4*, *IL10*, *SPRED2*, *STAT4*, *PXK*, *AFF1*, *IL12A*, *BANK1*, *TCF7*, *SKP1*, MHC genes, *IKZF1* and *IKZF3*, *BLK*, *ARID5B*, *CD44*, *LYN*, *ETS1*, *FLI1*, *SH2B3*, *CSK*, *ELF1*, *CIITA*, *ITGAM*, *TYK2*	*IKZF2*
IFN or Toll-like receptors	*IFIH1*, *PRDM1*, *UHRF1BP1*, *TNFAIP3*, *IRF5-TNPO3*, *IRF7* and *IRF8*, *SOCS1*, *PRKCB*, *UBE2L3*, *IRAK1*	None
Inflammation	*TNIP1*	None
Immune complex or waste clearance	*FCGR2A*, *FCGR2B*, *FCGR3B*, *ATG5*, *CLEC16A*	*NCF2*, *LYST*
Unknown	*ABHD6* (may be related to lymphocyte activation), *RAD51B* (may be related to IFN pathways), *MECP2* (may be related to IFN pathways), *RASGRP3*, *TMEM39A*, *PITG1*, *TNXB*, *JAZF1*, XKR6, *FAM167A–AS1*, *WDFY4*, unknown genes: rs1167796, rs463128, rs7186852, rs7197475	*SMG7* (may be related to interferon pathways), *DHCR7*, *NADSYN1*, *SLC15A4*, *PLD2*, *CXorf21*

GWAS, genome-wide association studies; IFN, interferon; MHC, major histocompatibility complex; SLE, systemic lupus erythematosus.

DNA methyltransferase 1 (DNMT1), an enzyme that creates epigenetic changes by DNA hypomethylation[65]. The influence of miRNAs was demonstrated when a transgenic mouse overexpressing miR-17-92 spontaneously developed lupus-like disease. The mechanism seemed to be diminished expression of T_{FH} cell regulators phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN and PH domain leucine-rich repeat-containing protein phosphatase 2 (REFS 66,67). Deficiency of miR-155 in MRL/lpr mice suppresses lupus, indicating that the effects of miRNAs are context-specific and site-specific, as would be expected[68]. In addition to studying the role of miRNAs in the pathogenesis of SLE, researchers are showing increasing enthusiasm for using these stable nucleic acids as biomarkers. For example, miR-21 regulates lymphocyte signalling, and levels of miR-21 in T cells correlate with SLE disease activity index (SLEDAI) scores[69]. The first miRNA-based therapeutic agent was approved in 2013 for the treatment of familial hypercholesterolaemia[70], and this field is likely to expand rapidly.

Differences in gene expression

Transcript abundance represents the final balance between active transcription and mRNA turnover, and integrates both transcript production and destruction effects. Levels of transcripts ultimately control cell activities. Early studies of gene expression were performed on peripheral blood mononuclear cells or whole blood and uniformly identified a set of ISGs[71–73]. Inflammatory and granulocyte signatures were also seen. These pivotal studies, now over ten years old, led to focused efforts on understanding the role of type I IFN and neutrophils. Gene expression has been examined in sorted cells from patients of varied ancestry[74]; ISGs could be identified in each cell population but the expression of specific genes varied dramatically between cell types, as well as between people of different ancestry[74]. This study is an important reminder that our current understanding of ethnic and population differences is disappointingly rudimentary. Array studies on human T cells have shown changes in gene expression related to disease activity and clinical presentation[75–77]. In oncology, arrays and other measures of gene expression are now routinely used to stratify patients' level of risk and direct therapy. Their use in rheumatology has been limited to research efforts, but a study utilizing advanced informatics found clear disease activity profiles[78]. Clinical use of gene expression for disease profiling could, therefore, become a reality in rheumatology clinics.

Apoptosis and nucleic acid sensors

Aberrant apoptotic cell clearance

The dysregulation of apoptosis and nuclear debris clearance that is characteristic of SLE contributes to an increase in autoantigen exposure[3]. The imbalance in apoptotic cell production and clearance is highly influenced by infection, ultraviolet light exposure, and cytokines. Accumulated apoptotic debris can trigger TLRs and nucleic acid sensors. Immune cells, including B cells, some T cells, dendritic cells (DCs), and macrophages, as well as nonimmune cells, such as epithelial cells and fibroblasts, express TLRs. Several pathways have evolved to prevent immune activation in response to endogenous cellular debris. Apoptotic cells become coated with complement component C1q, C-reactive protein, pentraxin 3, and serum amyloid P, which enhances phagocytosis without immune stimulation[79,80]. Additionally, DNase I contributes to degradation of chromatin[79]. Decreased DNase I activity has been described both in patients with SLE and in lupus-prone mice[81]. Characterization of the pathogenesis of monogenic forms of SLE has emphasized the role of aberrant apoptotic clearance. Sequencing analysis of seven consanguineous families with highly penetrant, autosomal recessive lupus-like disease identified inactivating mutations in *DNASE1L3* (REF. 82). Another family with a Mendelian pattern of SLE inheritance was found to carry loss-of-function mutations in *PRKCD*, which encodes the enzyme protein kinase Cδ[83]. This enzyme is activated in multiple apoptotic pathways. These rare monogenic diseases represent useful models of SLE because the disease process can be clearly defined. Although affected patients often have a phenotype that is not typical of classic SLE, they provide important insights.

Role of TLRs

Apoptotic cells are cleared largely by cells in the reticuloendothelial compartment. Clearance is generally silent but when the burden of apoptotic cells exceeds that which can be cleared, the apoptotic debris can elicit immune responses[84]. Mouse models have been instrumental in defining the role of TLRs in lupus; however, extrapolation of findings from these models to humans is controversial because not all features are consistent with our current understanding of SLE in humans. Nevertheless, in the analysis of specific pathways, mouse models of lupus offer great advantages. Collectively, they have provided confirmation of the importance of TLR trafficking.

Table 2 | **Monogenic causes of SLE and lupus-like disease**

Gene	Effect	Features	Pathway	Refs
C1QA, C1QB, C1QC	Complement C1 deficiency	Early-onset, severe SLE, infections; high penetrance*; AR	Immune complex and waste clearance	210,211
C1R, C1S	Complement C1 deficiency	Early-onset, severe SLE, infections; high penetrance; AR	Immune complex and waste clearance	212,213
C4A, C4B	Complement C4 deficiency	Early-onset, severe SLE, infections; high penetrance; AR	Immune complex and waste clearance	214
C2	Complement C2 deficiency	Infections, cutaneous disease; moderate penetrance; AR	Immune complex and waste clearance	215
C3	Complement C3 deficiency	Membranoproliferative glomerulonephritis; low penetrance; AR	Immune complex and waste clearance	216
CYBB	X-linked chronic granulomatous disease	Infections, chronic granulomatous disease; low penetrance; X-linked	Immune complex and waste clearance	217
PEPD	Xaa-Pro dipeptidase deficiency	Cutaneous ulcers; low penetrance; AR	Immune complex and waste clearance	218
MAN2B1	Lysosomal α-D-mannosidase (laman) deficiency	Hearing loss, dysostosis multiplex, progressive cognitive decline; low penetrance; AR	Lysosomal oligosaccharide catabolism	219
TREX1	Aicardi–Goutières syndrome 1	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR or AD	Nucleic acid sensing; type I IFN	220,221
DNASE1	SLE	High penetrance; AD	Nucleic acid sensing	222
DNASE1L3	SLE 16	Early onset; high penetrance; AR	Nucleic acid sensing	82
SAMHD1	Aicardi–Goutières syndrome 5	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR	Nucleic acid sensing; type I IFN	223
ACP5	Spondyloenchondrodysplasia with immune dysregulation	Spondyloenchondrodysplasia, vitiligo, growth retardation; low penetrance; AR	Nucleic acid sensing; type I IFN	224
RNASEH2A, RNASEH2B, RNASEH2C	Aicardi–Goutières syndrome 4, 2, and 3 respectively	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR	Nucleic acid sensing; type I IFN	225
ADAR	Aicardi–Goutières syndrome 6	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR or AD	Nucleic acid sensing; type I IFN	226
IFIH1	Aicardi–Goutières syndrome 7	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AD	Nucleic acid sensing; type I IFN	225
DDX58	Singleton–Merten syndrome 2	Dental loss, arterial calcification, joint contractures; high penetrance; AD	Nucleic acid sensing; type I IFN	227
TMEM173	STING-associated vasculopathy, infantile-onset	Skin ulcers, interstitial lung disease; low penetrance; AD	Nucleic acid sensing; type I IFN	228
ISG15	Immunodeficiency 38, with basal ganglia calcification	Mycobacteria, intracranial calcification; low penetrance; AR	Nucleic acid sensing; type I IFN	229
PSMB8	Nakajo syndrome	Fever, contractures, neutrophilic dermatitis; low penetrance; AR	Immune complex and waste clearance; type I IFN	230
FAS, FASLG	Autoimmune lymphoproliferative syndrome 1A and 1B, respectively	Autoimmune cytopenias, adenopathy; high penetrance; AD	Lymphocyte signalling	231–233
PRKCD	Autoimmune lymphoproliferative syndrome 3	Autoimmune cytopenias, adenopathy; moderate penetrance; AR	Lymphocyte signalling	83
PTPN11	Noonan syndrome 1	Short stature, cardiac anomalies; low penetrance; AD	Lymphocyte signalling	234
RAG1, RAG2	Several types of severe combined immune deficiency	Infections, granulomas; low penetrance; AR	Lymphocyte signalling	235,236

*Penetrance is indicted as a qualitative assessment of the percentage of people with the condition who have features of SLE.**AD, autosomal dominant;** AR, autosomal recessive; IFN, interferon; SLE, systemic lupus erythematosus.

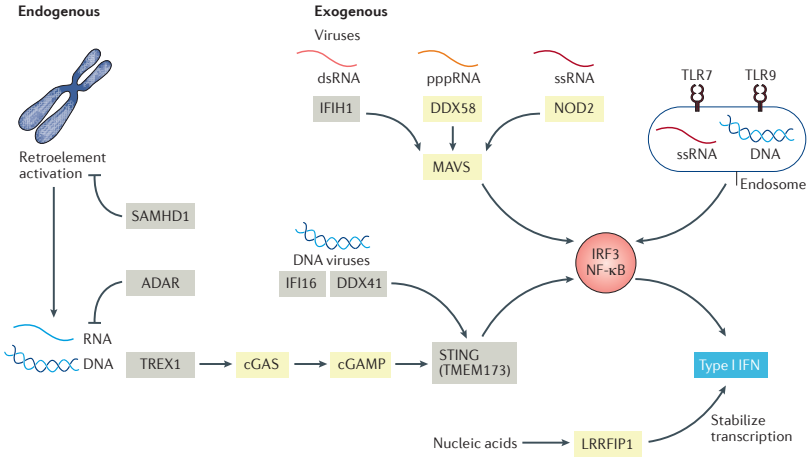

Figure 3 | **Nucleic acid sensors in SLE.** The importance of the immune response to nucleic acids in systemic lupus erythematosus (SLE) has been emphasized by data from mouse models and patients with monogenic diseases associated with defects in these pathways. Toll-like receptors (TLRs) are restricted to vesicles and primarily respond to endocytosed nucleic acids. Cytoplasmic sensors recognize endogenous nucleic acids as well as a myriad of viruses. Responses converge on two transcription factors, interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB), which are responsible for the induction of type I interferon (IFN) expression as well as some inflammatory cytokines.

TLR3, TLR7, TLR8 and TLR9 reside in the endoplasmic reticulum. Transfer of TLRs to endosomes is regulated by the trafficking protein unc-93 homologue B1 (UNC93B1). The importance of intracellular trafficking cannot be overstated, as it represents a key regulatory strategy. In plasmacytoid DCs (pDCs), UNC93B1 sorts large complexes of DNA into early endosomes, where TLR9 and IRF7 drive a strong IFN response. Small monomeric DNA is sorted into late endosomes, where TLR9 and NF-κB drive a proinflammatory cytokine response[85]. Correct localization of TLRs limits their access to self-antigens[86]. In pristane-treated mice, TLR7 (which senses single-stranded RNA) was specifically required for the production of RNA-reactive autoantibodies and for the development of glomerulonephritis[87]. Data from studies of pharmacologic or genetic manipulation of TLR7 expression or function support a central role for TLR7 in inflammation, loss of tolerance, and type I IFN production[88–91].

The relationship of TLR9 to SLE is more complex than that of TLR7. TLR9 is a receptor for DNA containing unmethylated CpG sequence motifs. SLE patients with active disease had a higher number of TLR9-expressing B cells and monocytes than did patients with low disease activity, and levels of these cells correlated with levels of antibodies to double-stranded DNA (anti-dsDNA)[92]. In TLR9-deficient lupus-prone mice, the generation of anti-dsDNA and anti-chromatin autoantibodies was specifically inhibited, while levels of other autoantibodies (such as anti-Sm) were maintained or even increased[93]. However, in one lupus model, TLR9 deficiency exacerbated disease through a mechanism that might relate to competition with TLR7 for UNC93B1 (REF. 94). TLR3 and TLR8 also recognize RNA and limited data support a role for these additional receptors in susceptibility to SLE. These data have led to a model of SLE in which TLRs engage nucleic acids and drive a type I IFN response (FIG. 3).

Cytosolic nucleic acid sensors

Cytosolic nucleic acid sensors recognize viral infections and initiate defences focused on type I IFN production. These sensors can also detect endogenous ligands and elicit inflammation independent of infection. Signalling pathways for these sensors converge on stimulator of IFN genes protein (STING, encoded by *TMEM173*)[95]. Additional protection from the deleterious effects of endogenous nucleic acids comes from nucleases, which degrade nucleic acids. Three cytosolic RNA helicases have been identified: probable ATP-dependent RNA helicase DDX58, interferon-induced helicase C domain-containing protein 1 (IFIH1, also known as MDA5), and probable ATP-dependent RNA helicase DHX58 (also known as LGP2). These sensors act in the cytoplasm to complement the function of endosomal TLRs[96]. The cytosolic sensors activate both IFN and inflammatory cytokine production[96]. Variants in *IFIH1* have been linked to SLE[97]. Cytosolic DNA sensors also exist. All three main types of inflammasomes can respond to DNA; however, the process that drives these responses is not well understood (with the exception of the AIM2 inflammasome, which is activated by STING)[98]. Mouse models of lupus support a key role for this pathway in the aetiopathogenesis of SLE[99].

Here again, extraordinary insights have come from the study of rare monogenic disorders with a lupus-like phenotype in humans. Aicardi–Goutières syndrome has features that are reminiscent of a congenital infection; however, this syndrome is caused by gene defects that drive overproduction of type I IFN. Specifically, mutations in genes encoding cytosolic nucleic acid sensors or their regulators, such as *TREX1*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *SAMHD1*, *ADAR*, or *IFIH1*, are all associated with this phenotype[100] (TABLE 2). The Aicardi–Goutières phenotype has a more prominent neurologic component than is typical of adult-onset SLE; however, autoantibodies are prolifically produced and some pathologic features overlap with those of SLE[100,101]. In addition, common variants in these same genes have been associated with SLE (TABLE 1).

Soluble mediators

Cytokines can contribute to susceptibility to SLE, but are more strongly implicated in loss of tolerance and end-organ effects (FIG. 1). Levels of many cytokines are elevated in SLE (such as TNF, IL-4, IL-6, and IL-10) and their main effects are the promotion of autoantibody production and inflammation (FIG. 4). Type I and type II IFNs have emerged as key cytokines in the pathogenesis of SLE (as well as other autoimmune diseases) and increases in their levels precede autoantibody development[102,103]. Upregulation of TNF can increase type I IFN expression[104,105]. IFNα, a type I IFN typically produced as part of the innate immune response to viral infection, has multiple effects consistent with known immunologic features of SLE, such as upregulation of B-cell activating factor (BAFF, also known as TNF ligand superfamily member 13B or BLyS), decreased T_{reg} cell function, and induction of plasma cells[106]. Transcripts of IFNα and ISGs have been detected in inflamed kidney and skin tissues from patients with SLE[107,108]. A direct pathogenic role for IFN in mouse models of lupus is also supported by studies in which exogenous administration of IFNα exacerbates disease[109,110]. Unfortunately, despite these compelling data, clinical trials of IFN inhibitors have been disappointing. Levels of two other cytokines, IL-18 and IL-38, are also increased in SLE. IL-18 is a potent proinflammatory cytokine produced via the inflammasome, and IL-38 is thought to be an anti-inflammatory cytokine with key regulatory functions[111,112].

Patients with SLE may also have an imbalanced T cell cytokine profile characterized by decreased IL-2 and increased IL-17 levels[113]. Production of IL-2 is impaired on multiple levels[114,115]. IL-2, in addition to being critical for T_{reg} cell development and function, is also necessary for restricting expression of IL-17. In SLE, IL-17 may mediate local tissue damage through the induction of inflammatory cytokines and chemokines, and by recruiting other immune cells. The differentiation of the T helper cell subset producing IL-17 (T_H17 cells) is dependent on IL-23, and an anti-IL-23 antibody ameliorated disease in one mouse model of lupus[116].

B-cell activation and autoantibody production are promoted in SLE by BAFF. Serum levels of BAFF are increased in patients with SLE and positively correlate with autoantibody titres[117]. Transgenic overexpression of BAFF in a mouse model of lupus exacerbated disease[118], emphasizing the role of this cytokine in supporting autoimmunity. BAFF is a critical factor for B-cell homeostasis and high BAFF levels might reduce the stringency of B-cell selection, allowing autoreactive clones to persist in the periphery[119]. Notably, B-cell-depletion therapy in patients with SLE is followed by an increase in BAFF levels, raising concern that the repopulating B cells could have a phenotype of increased autoreactivity[120]. BAFF thus represents an important therapeutic target; indeed, belimumab, an anti-BAFF monoclonal antibody, is the first drug to be approved for the treatment of SLE in more than 50 years[121]. BAFF-directed therapy has demonstrated clinical efficacy but the magnitude of the beneficial effect is modest, as has been true for B-cell-depleting approaches[122,123]. The message might be that narrowly targeted approaches in humans with established disease cannot reverse pathologic downstream processes that have

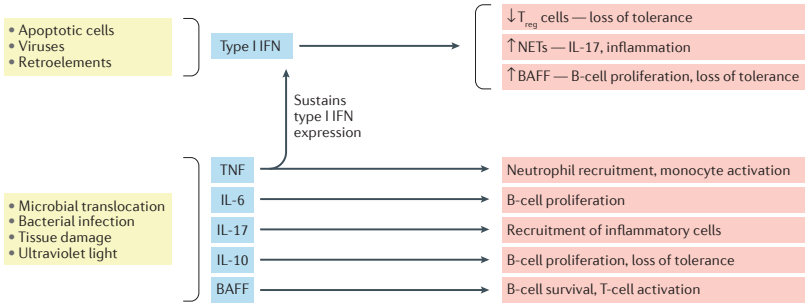

Figure 4 | **Cytokines implicated in SLE.** Various stimuli that have been epidemiologically associated with systemic lupus erythematosus (SLE) can drive cytokine expression. Collectively, the effects of this increased cytokine expression include both inflammation and loss of tolerance. BAFF, B-cell activating factor; IFN, interferon; NET, neutrophil extracellular trap; T_{reg} cell, regulatory T cell.

previously been initiated. Targeting of other cytokines is nonetheless a priority for the pharmaceutical industry because therapeutic monoclonal antibodies are an established pipeline.

Major cell types involved in SLE

Dendritic cells

Inappropriate or dysfunctional antigen presentation by DCs might promote the breakdown of T-cell and B-cell tolerance in SLE and other autoimmune diseases (FIG. 2). Patients with SLE show multiple DC abnormalities, including a reduced number of circulating conventional DCs, but increased numbers of pDCs[124]. The pDC subset is the primary cell type responsible for type I IFN secretion in response to nucleic acid, via TLR7 and TLR9. The pDCs take up immune complexes via FcγRIIa and access TLR7 and TLR9 in the endosomal compartment[125]. In SLE, conventional DCs promote autoreactivity rather than tolerance[126]. In turn, activated T cells also promote increased IFN production by pDCs[127]. Conventional DCs have been demonstrated to be critical for the development of lupus nephritis in a mouse model[128]. Thus, both types of DCs are thought to be pivotal to the disease process in SLE.

Myeloid cells

Neutrophils show several facets of dysregulation in SLE. Impaired phagocytosis by neutrophils in SLE has been described in multiple reports, and might contribute to the increased susceptibility to infection associated with this disease[129]. In one study, neutrophils from patients with SLE showed reduced production of reactive oxygen species (ROS), which correlated with disease severity and end-organ damage[130]. Patients with chronic granulomatous disease, in which ROS production is defective, have a high incidence of SLE[131,132] (TABLE 2). Increased levels of ISG products, autoantibodies, and glomerulonephritis have been described in a mouse model of chronic granulomatous disease, and lupus-prone mice deficient in ROS production also show an exacerbation of lupus-like disease[133,134]. Deficient ROS generation might alter the apoptotic pathway, which connects this finding to the recognized contribution of defective clearance of apoptotic cells to the pathogenesis of SLE. Immune complexes can drive the generation of mitochondrial ROS, and oxidized mitochondrial DNA can be highly immune-stimulatory, providing a feed-forward loop[135]. Neutrophils are short-lived and so represent the dominant cell type in the daily burden of apoptotic cells. Small changes in neutrophil apoptosis could markedly impact waste clearance. In an adoptive cell transfer model, neutrophils from mice with chronic granulomatous disease could drive autoantibody production in control (disease-free) recipient mice[136]. The converse was also true; apoptotic neutrophils from control animals were capable of driving autoantibody production when transferred to recipients with chronic granulomatous disease. This study provides direct mechanistic evidence for a central role of myeloid cells in SLE.

Patients with SLE have an abnormal subset of neutrophils (termed low-density granulocytes) with an increased propensity for NETosis[137]. NETosis is a mechanism of cell death that occurs in response to various stimuli, including infectious organisms and oxidative stress. NETosis involves the extrusion of chromatin and other nuclear, cytoplasmic, and granular material from the cell (FIG. 2). This extruded material, called neutrophil extracellular traps (NETs), contains proinflammatory cytokines, antimicrobial peptides, enzymes such as myeloperoxidase, and potentially antigenic citrullinated histones and dsDNA[138]. NETosis contributes to the type I IFN signature of SLE by stimulating IFN production by pDCs[137]. This effect occurs via TLR9 activation by DNA and anti-DNA antibodies in complex with NET-derived antimicrobial peptides such as cathelicidin antimicrobial peptide (also known as LL-37)[139,140]. In turn, type I IFN primes neutrophils for NET release in patients with SLE, suggesting a possible positive feedback loop. The extruded nuclear material from NETs represents a major source of the nuclear antigens that drive autoantibody development in SLE.

Monocytes from patients with SLE consistently have increased baseline expression of CC chemokine ligand 2 (CCL2, also known as monocyte chemoattractant protein 1 (MCP-1))[141]. MCP-1 is regulated by lipopolysaccharide and IFNs, and is important in regulation of cell migration. Monocyte infiltration into kidneys influences renal damage, and monocyte infiltration into blood vessels contributes to atherosclerosis, two key morbidities in SLE[142,143]. Renal macrophage infiltration is a particularly strong prognostic biomarker for progression of lupus nephritis[144]. Monocytes are, therefore, a pivotal cell type in organ damage. Monocyte-depletion therapy was attempted in one clinical trial, which did not demonstrate clinical effectiveness, but this approach did not deplete tissue macrophages, which are thought to be important drivers of end-organ damage in SLE[145].

T cells

T cells are thought to be central to the pathogenesis of SLE because of their association with MHC proteins, and because adoptive transfer of these cells confers lupus-like disease in some mouse models. Loss of T-cell tolerance is implied in autoimmune diseases. Conceptually, this loss of tolerance could happen centrally at the time of thymic education or peripherally; however, mouse models support the importance of defects in peripheral tolerance[146]. Deficient or defective T_{reg} cells have been identified both in mouse models and human studies[147]. GWAS have also identified defects in lymphocyte signalling that could centrally alter thymic deletion of autoreactive cells. Thus, multiple pathways exist by which T-cell tolerance could be defective in SLE. One of the first phenomena to be described was that of aberrant signalling through the T-cell receptor. This phenomenon is not cell-intrinsic, and can be induced in normal T cells by serum IgG from patients with SLE[148]. In T cells from patients with SLE, the CD3 ζ chain (which mediates signalling via tyrosine-protein kinase ZAP-70) is downregulated owing to increased mTOR activity, causing ZAP-70 to be replaced by FcRγ. FcRγ then pairs with tyrosine-protein kinase SYK rather than with ZAP-70, resulting in hyperactivation of the T-cell-receptor signalling pathway[149,150].

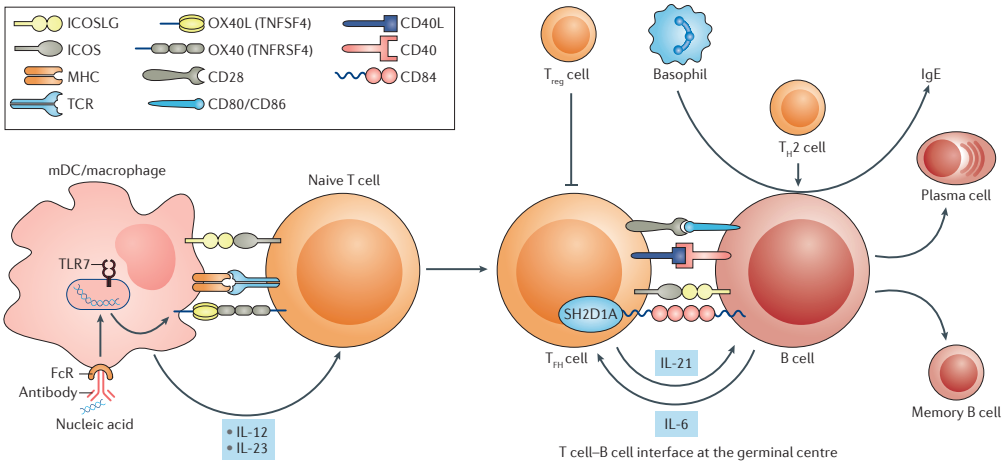

Figure 5 | **Involvement of B cells in SLE.** B cells are greatly influenced by the cytokine milieu and the type of T cell that derives from that cytokine milieu. In systemic lupus erythematosus (SLE), B cells interact with T follicular helper (T_{FH}) cells at the T cell–B cell interface in secondary lymphoid organs. The interaction revolves around engagement of cell-surface receptors and secretion of cytokines. FcR, Fc receptor; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; TCR, T-cell receptor; T_H2, type 2 T helper cell; TLR, Toll-like receptor; T_{reg} cell, regulatory T cell.

Despite this hyperactivated phenotype, T-cell production of IL-2 is actually impaired. Expression of IL-2 in SLE T cells is compromised by decreased levels of the transcription factor AP-1 and suppression by cAMP-responsive element modulator (CREMα)[114,115]. Treatment with the mTOR inhibitor rapamycin *in vitro* reversed this effect, and mTOR inhibitor treatment *in vivo* has been clinically efficacious, supporting the importance of this pathway in SLE[151].

Patients with SLE also show altered T-cell subset populations. T_H17 cells are a subset of $CD4^+$ T cells found infiltrating the kidneys of patients with lupus nephritis, and in the skin lesions of patients with SLE[152]. Polarization to T_H17 involves changes to the epigenome that can be driven by microbial products[153]. Double-negative T cells ($CD4^-CD8^-$) seem to be the primary source of IL-17 in SLE[154]. Double-negative T cells are expanded in patients with SLE as well as in lupus-prone mice and are thought to contribute to loss of tolerance[155,156], as they also express IL-1β and IFNγ, and promote B-cell differentiation and antibody production.

T cells provide more than just signals for class switching. They represent a key checkpoint for autoreactive B cells in SLE. T-cell–B-cell interactions are a key focus of current SLE research because these interactions occur outside their usual locations, in secondary lymphoid organs, and are more transient than in healthy individuals, suggesting that the very essence of the interaction is pathologic[157,158]. These aberrant T-cell–B-cell interactions are also reflected in the somatic mutations seen in autoantibody gene segments[159]. Somatic mutations reflect both T cell help and germinal centre passage. T_{FH} cells specifically support B-cell differentiation by producing IL-21 and receptor engagement in the germinal centre (FIG. 5). Expansion of the T_{FH} cell subset has been described in several mouse models of lupus[160,161] and increased levels of T_{FH} cells correlate with increased disease activity and severity in patients with SLE[162–164]. T_{FH} cells can be seen within lymphoid aggregates in kidney biopsy samples from patients with active lupus nephritis, and activated T_{FH} cells correlate with autoantibody titres in these patients[165,166]. Emerging evidence suggests that the expansion of T_{FH} cells in SLE is directed by interaction with OX40 ligand (also known as TNF ligand superfamily member 4 (TNFSF4)), which is expressed on myeloid antigen-presenting cells[167]. In SLE, the expression of OX40 ligand on myeloid antigen-presenting cells is induced (via TLR7 activation) by circulating RNA-containing immune complexes[167] (FIG. 5). The pathologically expanded and activated T_{FH} cell compartment markedly affects B-cell differentiation. Enhanced antibody production and loss of tolerance are both expected in this setting.

T_{reg} cells have an important role in maintaining tolerance. Both T cells and B cells are subject to T_{reg} cell control. Normal development of T_{reg} cells (a subset of $CD4^+$ cells that inhibit and suppress autoreactive lymphocytes) is dependent on IL-2. Treatment of patients with SLE with low-dose IL-2 for 5 days caused a dramatic increase in peripheral blood $CD25^+FoxP3^+$ T_{reg} cells, although the clinical consequences of long-term IL-2 therapy have not yet been determined[168]. This study might be seen as an important proof of principle for *in vivo* T_{reg}-cell-directed therapy.

B cells and autoantibody production

Although SLE is a clinically heterogeneous disease, patients are near-universally characterized by the presence of autoantibodies, particularly those directed against nuclear antigens. Loss of tolerance and altered B-cell differentiation might be genetically determined, by variants present from birth or acquired as part of the disease process[169]. Activation of B cells through the TLR pathway promotes loss of tolerance. Mouse models have demonstrated that transitional B cells that have recently emigrated from bone marrow are susceptible to accelerated maturation by TLR9, which bypasses tolerance checkpoints[170]. Tolerance can also be broken by B-cell stimulation via cytokines; BAFF in particular has been implicated in this process. BAFF antagonism in mice clearly leads to improved self-tolerance, and conversely BAFF overexpression leads to autoimmunity[171–173]. Tolerance does not seem to be an all-or-nothing phenomenon, however. An elegant demonstration of the evolution of autoantibodies in SLE was performed using stored plasma from members of the armed forces. This study demonstrated progressive development of autoantibodies over the 5–8 years preceding onsert of the clinical manifestations of SLE[174]. Human studies have clearly implicated both environmental and genetic contributions in loss of tolerance. Early immature B cells show increased levels of polyreactivity and autoreactivity in SLE, possibly owing to a break in central B-cell tolerance that enables increased numbers of autoreactive clones to reach the periphery[175]. B-cell subsets are skewed to the more mature subsets, those poised to become antibody-secreting plasma cells[176]. In addition, IL-10-secreting B cells with regulatory capabilities show functional impairment in SLE[177,178]. These observations support the concept that B-cell development is aberrant in SLE.

B cells contribute to SLE through their responses to antigen, regulation of other cells, and autoantibody production. Autoantibodies contribute to SLE through the formation of immune complexes, direct agonist or antagonist action, and by interference with intracellular functions[179]. Immune complexes activate complement and, through binding Fc receptors, drive inflammation. A unique indirect mechanism of action occurs through binding of RNA. The 60 kDa SSA/Ro protein binds RNA, preferring Alu retroelement RNA. Anti-Ro antibodies deliver this Alu RNA to the endosomal compartment via Fc receptors, thereby activating TLRs[180]. Antibody production in patients with SLE in general seems to favour high-affinity versions, as even anti-influenza virus antibodies have higher affinity in patients with SLE than their counterparts in healthy controls do[181]. A previously unanticipated B-cell phenomenon is the production of pathologic IgE antibodies. IgE is typically associated with allergic responses, and little effort was made to characterize IgE in patients with SLE until researchers showed that half of SLE patients have IgE directed to dsDNA[182]. Levels of self-reactive IgE increase with increased disease activity in patients with SLE and the IgE immune complexes can stimulate type I IFN in pDCs[183]. High total IgE concentrations have also been described in patients with SLE[184], but even in the absence of high IgE levels, autoantibodies of the IgE isotype and dysregulated basophils have now been observed in both mouse models of lupus and patients with SLE[185]. High numbers of basophils in mouse models of lupus contribute to a T_H2 cell polarization[186]. Importantly, depletion of either IgE or basophils in mice with lupus led to diminished renal disease, supporting their mechanistic role in SLE[182,185–187] and providing support for a clinical trial of IgE-directed therapy.

Organ-specific disease features

New experiments highlight that loss of tolerance and tissue damage are two distinct processes. Autoimmunity and kidney damage in NZM2328 lupus-prone mice are controlled by variants in *Agnz1* and *Cgnz1*. Replacement of the pathologic *Cgnz1* allele with the normal allele did not affect the expression of autoimmunity, but prevented kidney failure[188]. In another example, when the *gld.apoE*$^{-/-}$ mouse (a lupus-prone mouse with profound atherosclerosis) was rendered IRF5-deficient, it was protected from autoimmunity but displayed increased numbers of atherosclerotic lesions[189]. Thus, tissue effects are regulated independently of tolerance. These local tissue effects, which are also independent of haematopoietic cell influence, are major contributors to end-organ damage in SLE. These effects have been best described for kidney, skin, and the central nervous system (CNS).

Nephritis

Among women with SLE, approximately 30–40% of those with European ancestry, and nearly 50% of those with Afro-Caribbean ancestry develop lupus nephritis, which is associated with substantial morbidity and mortality[190,191]. Central features are immune-complex deposition and cell proliferation. Anti-dsDNA antibodies crossreact with several renal cell types and are thought to be central to the nephritis process. GWAS identified a lupus-nephritis-associated variant near the gene encoding the platelet-derived growth factor (PDGF) receptor[192]. Expression of PDGF and its receptor is increased in kidney tissue from patients with SLE[193], and anti-PDGF antibodies inhibit mesangial cell proliferation in animal models[194].

HER2 (also known as ERBB2) is also overexpressed in lupus nephritis[61] and can be upregulated by IFNs and IRF1 (REF. 61). HER2 regulates miR-26a, which in turn regulates cell proliferation[195]. The HER2-miR-26a pathway may be of clinical interest because anti-HER2 agents have already been developed for breast cancer treatment. Mesangial cells are capable of producing IFNs, which may amplify local inflammatory processes[196], regulate HER2 expression, and inhibit renal progenitor cell differentiation into podocytes, which compromises healing. In turn, mesangial proliferation and podocyte function are controlled in SLE by local activity of calcium/calmodulin-dependent protein kinase type IV (CaMK IV)[197,198]. Treatment of MRL/lpr mice with a CaMK IV inhibitor decreased IFN production and ameliorated nephritis[199]. Local cytokine production is thought to amplify the cell infiltrate. In the MRL/lpr model, TNF and IFNγ are produced in glomeruli before active cellular infiltrate[200].

Thus, therapies that limit tissue damage by targeting renal parenchymal cells may also prove useful in the treatment of lupus nephritis.

Skin

Cutaneous involvement is common in SLE and skin can constitute the only organ affected. Skin lesions are seldom life-threatening, but represent an important source of morbidity in SLE. Different subsets of cutaneous lupus erythematosus (which have distinct natural histories) are classified as acute, subacute, discoid, and intermittent (lupus erythematosus tumidus). Ultraviolet light is a typical precipitant of an SLE flare as a result of keratinocyte apoptosis. Immune complexes can be seen in skin biopsies from patients with SLE (termed the 'lupus band') and this finding is in fact diagnostic of SLE.

Common autoantibodies seen in patients with cutaneous forms of SLE are anti-ribosomal P protein and anti-galectin-3. Antibodies to Ro52 (also known as TRIM21) are also found, and deficiency of Ro52 in mice induces a lupus-like skin disease[201]. Why the effects of Ro52 deficiency were localized to the skin is unclear, but Ro52 is highly expressed in inflamed skin[202], and this finding might reflect the role of Ro52 as a nucleic-acid-binding protein rather than as having a direct role in providing protection to the skin[203]. Cutaneous lesions in SLE might, therefore, reflect the presence of specific autoantibodies, but also seem to relate to the cutaneous-dominant expression of certain proteins.

CNS disease

CNS disease remains one of the most troubling and puzzling clinical features of SLE. A meta-analysis indicated that polymorphisms in genes associated with immune-complex clearance, such as *FCGR3A* and *FCGR3B* (encoding low affinity IgG Fc region receptors IIIa and IIIb (FcγRIIIa and FcγRIIIb)) and *ITGAM* (encoding integrin αM) are potential susceptibility genes for neuropsychiatric lupus[204]. Polymorphisms in *TREX1* (which encodes 3′ repair exonuclease 1, also known as DNase III), have also been associated with seizures in SLE[205].

Dysfunction of the blood–brain barrier enables immunoglobulins, cytokines, and immune cells to gain access to the brain tissue, and is a central mechanism of neuropsychiatric lupus. The complement system has a key role in disrupting the integrity of the blood–brain barrier. Treatment with a C5a receptor antagonist or a C5a antibody improved the function of the blood–brain barrier and decreased CNS inflammation in mouse models of lupus[206,207]. Complement inhibition also improved neuronal survival in these studies[206,207].

Autoantibodies, including antiphospholipid antibodies and those targeting ribosomal P peptides, the NMDA receptor, and matrix metalloproteinase-9, could participate in the pathogenesis of neuropsychiatric lupus through multiple mechanisms, including by directly causing neuronal cell death[208]. In MRL$^{lpr/lpr}$ mice, CNS disease was amplified by the cytokine TNF-related weak inducer of apoptosis (TWEAK, also known as TNF ligand superfamily member 12). Mice deficient in the TWEAK receptor had better cognition and integrity of the blood–brain barrier than their littermates[209]. These studies open the door for therapeutics for CNS disease, for which there is a critical unmet need.

Conclusions

Conventional therapy for SLE has utilized broad-based immunosuppression. Advances in our understanding of SLE pathogenesis, as described here, will enable the development of targeted therapies that may lead to individualized approaches to care. Many of the advances made over the past decade are driving interest in developing targeted therapeutics and repurposing of drugs. Cytokines, tolerance pathways, local tissue mediators, and epigenetic mechanisms show promise as novel targets in SLE.

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Author contributions

K.E.S. and P.C.R. reviewed and edited the manuscript before submission. G.C.T., M.S.L., and P.C.R. researched data for the article, wrote substantial sections of the manuscript, contributed substantially to discussions of the content, and reviewed the final draft.

Competing interests statement

The authors declare no competing interests.

Appendix B.2 – Paper ***The SLE Transcriptome Exhibits Evidence of Chronic Endotoxin Exposure and Has Widespread Dysregulation of Non-Coding and Coding RNAs***, published in *PLOS One*, May 2014.

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The SLE Transcriptome Exhibits Evidence of Chronic Endotoxin Exposure and Has Widespread Dysregulation of Non-Coding and Coding RNAs

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Abstract

Background: Gene expression studies of peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE) have demonstrated a type I interferon signature and increased expression of inflammatory cytokine genes. Studies of patients with Aicardi Goutières syndrome, commonly cited as a single gene model for SLE, have suggested that accumulation of non-coding RNAs may drive some of the pathologic gene expression, however, no RNA sequencing studies of SLE patients have been performed. This study was designed to define altered expression of coding and non-coding RNAs and to detect globally altered RNA processing in SLE.

Methods: Purified monocytes from eight healthy age/gender matched controls and nine SLE patients (with low-moderate disease activity and lack of biologic drug use or immune suppressive treatment) were studied using RNA-seq. Quantitative RT-PCR was used to validate findings. Serum levels of endotoxin were measured by ELISA.

Results: We found that SLE patients had diminished expression of most endogenous retroviruses and small nucleolar RNAs, but exhibited increased expression of pri-miRNAs. Splicing patterns and polyadenylation were significantly altered. In addition, SLE monocytes expressed novel transcripts, an effect that was replicated by LPS treatment of control monocytes. We further identified increased circulating endotoxin in SLE patients.

Conclusions: Monocytes from SLE patients exhibit globally dysregulated gene expression. The transcriptome is not simply altered by the transcriptional activation of a set of genes, but is qualitatively different in SLE. The identification of novel loci, inducible by LPS, suggests that chronic microbial translocation could contribute to the immunologic dysregulation in SLE, a new potential disease mechanism.

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Introduction

Systemic lupus erythematosus (SLE) is the quintessential systemic autoimmune disease. The etiopathogenesis is still not fully understood and there are over 20,000 published studies evaluating various aspects of cellular dysfunction in this disease. Over the past 10 years, insights have come from genome-wide association (GWA) studies as well as gene expression studies. In both cases, a type I interferon pathway was implicated [1,2].

One of the hallmarks of lupus is the presence of autoantibodies directed against nucleic acid targets and other nuclear antigens. The process of apoptosis exposes the immune system to nucleic acids and nuclear antigens, particularly when the apoptotic cells are not cleared appropriately and degrade into smaller components [3]. The apoptotic debris are believed to drive much of the type I interferon signature and the type I interferon itself can drive additional apoptosis. Nevertheless, much remains unknown about the pathogenesis of SLE, particularly at the level of nucleic acid accumulation and dysregulated gene expression. Dysregulated gene expression, with the accumulation of aberrant transcripts, could theoretically contribute to apoptosis or increased type I interferon expression and has been shown to mimic lupus [4,5,6,7,8,9].

We utilized next generation sequencing of transcripts (RNA-seq) to characterize the SLE transcriptome in monocytes. Monocytes are a critical cell in SLE. They are implicated in renal damage, which is the major cause of morbidity in SLE, and in atherosclerosis, which is the major cause of mortality in SLE [10,11,12,13]. Monocytes are, therefore, central to the disease

process, but are also of interest because they respond to environmental stimuli, alter their function accordingly, and reflect that information back to other immunologically competent cells. They offer the additional advantage of representing a relatively homogeneous population [14]. This is the first RNA-seq study of SLE and we found not only a transcriptome that exhibits quantitative alterations as defined by the level of gene expression, but also qualitative differences with widely altered splicing preferences and non-coding RNA transcription. Some novel transcripts expressed at higher abundance in SLE monocytes were inducible by LPS, known to activate type I interferons [15,16,17]. LPS and microbial products have been demonstrated to accelerate renal disease and induce lupus-like processes in mice [18,19,20,21]. This finding provides an additional perspective from which to understand SLE.

Methods

Patients and cell purification

Investigators at Johns Hopkins University (JHU) School of Medicine obtained written informed consent and HIPAA Authorization of study subjects for all SLE samples. The Institutional Review Board at Johns Hopkins reviewed and approved the study of SLE patients. The use of the anomyzed Red Cross samples was approved by the Red Cross Institutional Review Board. Control samples were obtained from The Center For Aids Research, which supplies blood samples on a fee for service basis. They have obtained consent for the use of the samples and their protocols were approved by the University of Pennsylvania Institutional Review Board.

Primary human monocytes were purified using elutriation and adherence from eight healthy controls and nine SLE patients with no other autoimmunity, as previously described [22,23,24,25]. The purity of monocytes was more than 90% by flow cytometry for CD14 staining. All controls and patients were female and an average of approximately 40 years of age. All SLE patients' disease activity was mild-moderate and no one was on high-level immune suppression (Table S1). All subjects were enrolled in an IRB-approved study and provided informed consent, except the red cross serum samples which were provided as anomyzed discarded samples.

RNA isolation and library preparation

Total RNA was isolated from 2–3 million primary monocytes using the Qiagen RNeasy kit and DNA was removed by on-column DNase digestion (Qiagen, Valencia, CA). This method recovers predominantly RNA species >200 bases. 1 μg of total RNA was used to prepare the library with the SOLiD™ whole transcriptome analysis kit (Applied Biosystems, Foster City, CA), providing strand-specific data. The procedure followed the instructions of the manufacturer. For miRNA validation, we used miRNeasy from Qiagen. Table S2 provides RNA quality and cell count information. The OD 260/280 ratio ranged from 1.8–1.98. RIN scores were not consistently obtained prior to fragmentation but were >7 for those where the RIN was defined. Post fragmentation, the RIN score averaged 2.3. The RNA quality and counts were not different between patients and controls.

RNA abundance validation

qRT-PCR was used to define quantitative differences in RNA abundance. The Clontech Advantage RT for PCR kit (Clontech, Mountain View, CA) was used to generate cDNA. Gene expression was detected by real-time PCR using the TaqMan 9600. Transcript levels were normalized to the 18S or β-actin signal, as has been previously used in SLE studies [26,27,28]. Mature miRNAs were detected with Taqman miRNA assays. Relative quantitation was performed using spiked *Caenorhabditis elegans* miRNA-238 as an exogenous control (Qiagen Syn-cel-miR-238-3p miScript miRNA Mimic). Commercially available primers were purchased from Applied Biosystems for: cel-miR-238 (248 primer for isoform cel-miR-238-3p - MIMAT0000293); hsa-miR-212 (515 primer for isoform hsa-miR-212-3p - MIMAT0000269 and 461768_mat for isoform hsa-miR-212-5p - MIMAT0022695); and *CCR2* (Hs00174150 primer for *CCR2* isoform NM_001123041.2 and Hs00704702_s1* for *CCR2* isoform NM_001123396.1) and from Qiagen for *RND3* (QT00002744),*TSLP* (QT01670809), *RGPD1* (QT01678425), *CD177* (QT02452849), *TUBB1* (QT00049574), and *ITG-B* (QT01003121). Novel loci were detected with custom primers using SYBR green. Primer sequences are listed in the Methods S1. The Mann Whitney U test was used to analyze the differences between SLE and controls.

Endotoxin analyses

MonoMac 6 cells and primary monocytes from healthy donors were stimulated with 100 U/ml α2-interferon (PBL Biomedical Laboratories, Piscataway, NJ), 10 ng/ml γ–interferon (R&D Systems, Inc., Minneapolis, MN), 10 ng/ml tumor necrosis factor (TNF-α) (Sigma, Saint Louis, MO) for 16 hours, or 1 μg/ml of LPS for two hours. SB203580 was used as a p38- MAPK inhibitor by pretreating the cells for 30 minutes at a concentration of 10 μM. SP600125 (Calbiochem, Darmstadt, Germany) was used as a JNK inhibitor at a concentration of 10 μM and U0126 (Cell signaling, Danvers, MA) was used as an ERK inhibitor at a concentration of 10 μM. Cells were harvested after stimulation and RNA was prepared as above. To measure circulating endotoxin, serum samples from 99 SLE patients and 112 Red Cross blood donors were analyzed using the Limulus assay (Thermo Scientific, Rockford, IL). The Wilcoxon method was used to compare the levels across groups.

Bioinformatics

We used the Tophat-Cufflinks pipeline and further refinements [29] to assemble the monocyte transcriptome and detect novel loci and isoforms, followed by mapping short reads to a collection of reference RNA sequences, including isoforms of coding genes, small RNAs, long non-coding RNAs (lncRNAs), and repetitive elements. The number of reads mapped to each transcript was used for evaluating differential expression between control and SLE samples. Data has been submitted to GEO as GSE53419.

The following steps were used in data analysis:

- Use TopHat to align 50 bp sequencing reads to reference genome hg19. TopHat also searched for reads partially mapped to distant locations to identify exon-exon junctions. As a result, TopHat mapped ~22 million reads per sample on average and stored the aligned reads in BAM files. This step did not use any known gene annotation
- Use Cufflinks to assemble transcriptomes of individual samples while using RefSeq gene annotation as reference. On average, Cufflinks reported 65,008 genes and 83,430 transcripts in individual transcriptomes.
- Use Cuffmerge to combine individual transcriptomes, also using RefSeq as reference. The merging improved the reliability of novel loci and isoforms by taking a consensus of individual transcriptomes. Cuffmerge reported 59,887 transcripts of 34,307 genes in a consensus transcriptome.

- Use Cuffcompare to categorize transcripts by comparing the consensus transcriptome to RefSeq annotation. For example, a class "=" transcript had the same exon-exon junctions as a known RefSeq transcripts and a class "u" transcripts had no overlapping to any known genes. As a result, Cuffcompare identified 10,200 novel transcribed loci and 10,313 isoforms including novel exon-exon junctions of known genes.
- Use the gene annotation tracks downloaded from UCSC Genome Browser to evaluate the novelty of the new loci and isoforms reported by Cuffcompare. The tracks were UCSC Genes, Ensembl Genes, GENCODE Genes V12, CCDS, H-Inv 7.0, sno/miRNA and lincRNA Transcripts, including evidence-based gene annotation, computationally generated gene prediction, and collections of non-coding RNAs. The comparison identified 2,280 and 4,000 high novelty loci and isoforms not included in any of the seven annotation tracks.
- Repeat the TopHat-to-Cuffcompare steps without using a reference annotation for assembling and merging transcriptomes. Cuffcompare identified 774 transcripts having the exact same exon-exon junctions as known RefSeq transcripts. These true positives had higher read counts and consistency across samples. Surprisingly, they were also longer and included more exons on average, which presumably made them more difficult to be completely assembled. High read count and high consistency were then used as the criteria to filter the novel loci and isoforms identified by previous steps to obtain 3,725 and 1,327 high-confidence novel loci and isoforms respectively.

Align reads to reference transcriptome using Novoalign. NovoalignCS was used to align reads to a reference transcriptome to obtain the read count per transcript. Each entry of the reference transcriptome was the full sequence of a transcript without introns. The reference transcriptome included all RefSeq transcripts and novel loci/isoforms reported by Cuffmerge, as well as three classes of non-coding RNAs: small RNAs from snoRNABase and miRBase), lincRNAs (UCSC Genome Browser lincRNAs track), and repetitive elements (Repbase).

Reads mapped to the same transcript were counted to define the transcription level. Reads mapped to different RNA classes or transcripts of different genes were excluded. The number of unique reads mapped to different transcripts of the same genes were used as gene-level read count. Only reads mapped to the sense strand were counted, whereas reads mapped to the antisense strand of coding genes were used to quantitate antisense transcription.

Statistical analysis of transcriptional data. We noticed that the fold change of patients and controls was slightly dependent on the baseline expression level ($r = 0.15$, $p < 0.01$), and applied Loess adjustment to remove this bias by assuming an equal amount of total RNA between samples. The global average of patient-control difference was very close to zero (0.4% higher in SLE) after the adjustment.

Statistical analysis of differential expression was performed within R environment using Bioconductor packages. Rsamtools package was used to import aligned reads from BAM files to R environment. Gene-level differential expression between SLE and control samples was analyzed with the Bioconductor EdgeR package. Genes without at least three read counts in at least three samples were excluded.

Gene-gene correlation analysis was performed by the following steps: calculate the correlation coefficients between genes in control and SLE groups separately; convert correlation coefficients to z scores using Fisher's transformation; take the average of z scores, and convert the average z score back to a single combined correlation coefficient r. Enrichment studies utilized DAVID which reports adjusted p values based on the Benjamini Hochberg algorithm [30].

3′ Untranslated analysis. For analysis of the 3′ untranslated regions, RNA-Seq reads were aligned to reference genome (UCSC, hg19) using Tophat 1.3.1 by default parameters for Applied Biosystems' Colorspace format. Uniquely mapped reads were kept for downstream analysis. PolyA_DB together with the 3′ ends of transcripts from UCSC, RefSeq, Aceview annotations were collected as a comprehensive APA database [31]. For each annotated cleavage site, the number of reads in the upstream 200 nt window were computed as the estimation for the expression level of the mRNA isoform terminating at that 3′end. The read count for each isoform was added up in nine case samples and eight control samples, respectively. We required at least 10 reads supporting each isoform resulting in ~6600 APA genes for analysis. The relative ratio of expression at proximal to distal sites, in addition with a p-value, was calculated by Fisher's Exact test using R (http://www.r-project.org/). Relative ratio>2 and p-value<0.01 were used to predict different isoform usage.

Analysis of published gene sets. Five data sets were created from four monocyte GEO data series, GSE15219, GSE19627, GSE5504, and GSE21909 (one from each of the first three and two from GSE21909, which used saline and cortisol for intravenous delivery of LPS). Samples and groups were selected so that each group had matched replicates/donors. (Table 1)

Results

Transcriptome characterization

Monocyte transcriptomes from Nine female SLE patients and eight female healthy controls were studied using RNA-seq. Total RNA was purified from monocytes and used for library preparation. Our first goal in this study was to reconstruct the human monocyte transcriptome using the entire dataset. The Tophat-Cufflinks pipeline reported four major classes of transcripts (Figure S1A), including 10,313 isoforms of known genes and 10,200 transcribed loci not included in the RefSeq annotation. The processed data are given in an excel spreadsheet in Data S1. The novel isoforms had a more complex structure (Figure S1B). On the other hand, the novel loci were shorter on average than known genes and over 97% of them included only one exon.

To further define the monocyte transcriptome, we counted the total number of reads solely aligned to each of the four major RNA classes: coding RNAs, long non coding (lncRNAs), small RNAs, and repetitive elements. Ribosomal RNAs (rRNA) were excluded from this summary. Close to 80% of the non-rRNA reads were mapped to coding RNAs while the other three classes evenly shared the remaining reads (Figure S1C). Small RNAs had the highest molar concentration after read counts were adjusted by the total length of the RNA classes, followed by repetitive elements, coding RNAs, and lncRNAs (Figure S1D). These data were comparable to the distribution seen in other systems [32].

The classifications of transcripts were refined. We identified 4,000 high-novelty isoforms that were not present in seven additional annotated databases and included at least one unknown exon-exon junction, as well as 2,271 high-novelty loci that had no overlap with either exons or introns of any previously reported transcripts. We then defined 1,327 high-confidence isoforms, having at least 10 uniquely mapped reads not mapped to any other genes or isoforms of the same gene in at least six samples, and 3,725 high-confidence novel loci having at least 10 uniquely mapped reads in at least six samples and more than 10× overall sequencing depth. After this refinement, 448 novel isoforms and

Table 1. GEO sources.

	# groups	# replicates
GSE15219	4	2
GSE19627	2	3
GSE5504	4	2
GSE21909_Saline	3	3
GSE21909_Cortisol	3	3

doi:10.1371/journal.pone.0093846.t001

778 novel loci that had both high-novelty and high-confidence were identified. There was no association between high-novelty and high-confidence (Figure S2). We manually examined a subset of the 778 novel loci for protein coding potential and the possibility that they are orthologs of coding genes in other species using BLAST. Most did not exhibit similarity to any annotated genes and the majority had limited protein coding potential (<100 contiguous amino acids). We selected 27 novel loci and validated transcription from all 27 using RT-PCR (Figure S3, Table S3).

Class-specific transcripts in SLE

The major goal of this study was to identify a unique signature of the monocyte transcriptome in SLE as a strategy to improve our understanding of the pathogenic mechanisms. We found that there were a large number of known protein coding genes expressed in normal monocytes, but silenced in SLE (Figure 1A). These genes were highly enriched with ones related to embryo development (p = 6.0E-60), suggesting that SLE monocytes are more differentiated. Antisense transcripts were also more likely to be silenced in SLE (Figure 1B). On the other hand, the numbers of novel loci and novel isoforms transcribed only in patients were 3.95 and 1.49 times higher, respectively, than the numbers of control-specific ones (Figure 1C and 1D).

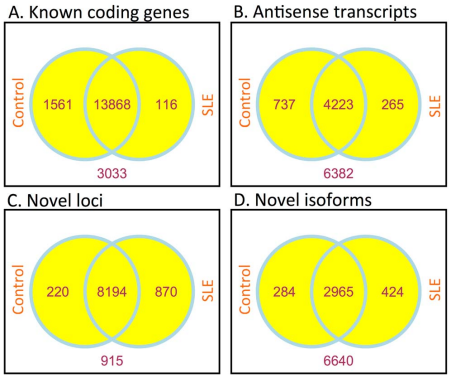

Figure 1. Comparison of SLE and control transcriptomes. The Venn diagrams demonstrate the overlap of actively transcribed A) coding genes, B) antisense transcripts, C) novel loci, and D) novel isoforms in the two sample groups. Eight control and nine SLE libraries were used.
doi:10.1371/journal.pone.0093846.g001

We evaluated the differential expression between the two groups of samples in six classes of genes/transcripts: known RefSeq protein coding genes, novel loci identified by Cufflinks, lncRNAs, small RNAs, repetitive elements, and the antisense transcripts of coding genes. These genes/transcripts were filtered by a statistical analysis, so all the remaining 33,760 (72% of 47,171 total reads) were mapped by at least three sequencing reads in at least three samples. We applied the negative binomial test implemented by the edgeR package of Bioconductor [33] and identified 1,754 differentially expressed genes/transcripts with p values less than 0.01 and a false discovery rate (FDR) of 0.2. Classes of differentially expressed genes (DEGs) are shown in Table S4.

The patient-control difference of total transcription varied dramatically between RNA classes (Figure 2A). Total transcription of both sense and antisense transcripts of known coding genes was reduced by approximately 15% in SLE (p = 5.6E-61 and 7.4E-105 respectively). Total transcription of novel loci was strikingly increased by over 45% in SLE (p<1.0E-300), as SLE patients produced many previously undiscovered transcripts that had low or no transcription in healthy monocytes. Although expression of small RNAs was downregulated by about 5%, expression of 91 pri-miRNAs was dramatically increased by 38% (p = 0.01), whereas the three classes of small nucleolar RNAs were all downregulated in SLE patients (Figure 2B). The overall up-regulation of pri-miRNAs and down-regulation of coding genes jointly suggested a modified miRNA regulatory system in SLE. Subclasses of repetitive elements also had different directions of change in SLE (Figure 2C). All endogenous retroviral (ERV) subclasses were consistently downregulated (p = 4.7E-4 to 3.8E-29). Other retrotransposons such as LTR retrotransposons, SINE, and LINE (L1) elements were variably decreased in SLE patients, while expression of SINE1/7SL elements was increased.

Protein-coding genes

Functional analysis of the protein coding genes identified a large number of pre-defined gene sets whose members were enriched within differentially expressed genes (DEGs), partially listed in Table S5. Most noticeably, DEGs upregulated in SLE were enriched with genes related to immune response and cytokine activity, while the downregulated DEGs were enriched with genes related to cell adhesion and motion. According to Genetic Association Database (GAD), eleven upregulated DEGs were associated with SLE in previous studies (Table S6). In addition, the combined gene set of up- and downregulated DEGs was enriched with potential targets of transcription factors highly relevant to SLE, including AP1 (p = 3.4E-09), E47 (p = 1.1E-8), RFX1 (5.8E-7), IRF1 (p = 1.4E-3), and IRF2 (p = 1.3E-3). We selected six DEGs, five upregulated and one downregulated in SLE, to be validated in new samples using qRT-PCR. Five of the genes were significantly different between controls and patients (p = 0.049 to

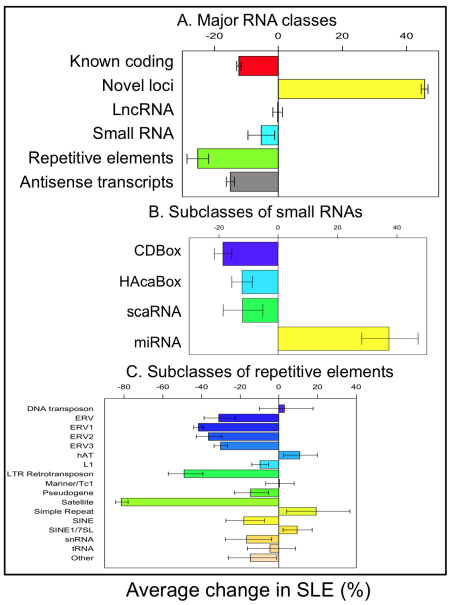

Figure 2. Differential expression of RNA classes. A) The average transcriptional changes of five RNA classes in SLE. B) The average transcriptional change of four sub-classes of small RNAs in SLE. C) The average transcriptional change of 17 subclasses of repetitive elements in SLE. The eight control and nine SLE libraries were used for this analysis. Error bars indicate standard deviation.
doi:10.1371/journal.pone.0093846.g002

0.0001) and all six were changed in the same direction in both patient cohorts (Figure S4).

Polyadenylation

Polyadenylation along with mRNA cleavage and termination are regulated in a gene-specific manner and these steps appear to be particularly important for the regulation of genes involved in inflammation [34,35,36]. Tissue-specific and developmentally-regulated polyadenylation have been described with differentiation favoring longer 3′ UTRs [37,38,39]We examined the structure of the coding genes by defining the 3′ untranslated region for each gene. Sixty-seven genes had longer 3′UTRs in patients compared to controls and 54 genes had shorter 3′UTRs in patients (defined by a ratio of >2.0 and a $p<0.01$). The longer 3′UTR gene set was characterized by pathways centered on NFκB, Akt, UBC and HNF4A. The shorter 3′UTR gene set was characterized by pathways centered on UBC, NFκB, and ERK (Figure S5). As shorter 3′UTRs can escape regulation by miRNAs and the inflammatory pathways implicated by the pathway analysis are concordant with what is known about SLE, these gene sets are notable and may given insight into the SLE process [38,40].

Antisense transcription

We analyzed antisense transcription because of the potential for sense-antisense duplex RNA to drive type I interferon expression and because antisense transcripts can regulate transcription in *cis* [41]. The gene-level read counts of sense and antisense transcripts were positively, but weakly, correlated ($r = 0.19$, $p<0.01$). Overall, examining patients and controls together, the total number of reads mapped to the antisense transcripts was about 1/30 of the total number of reads mapped to the sense transcripts. However, a small set of genes demonstrated much higher antisense transcription than sense transcription (Figure S6), such as *USP5*, a ubiquitin peptidase, and *CMTM5*, a chemokine-like factor gene.

In SLE, some coding genes had significantly changed antisense transcription in the opposite direction of their sense counterparts, most noticeably *IVNS1A BASES* (influenza virus NS1A binding protein), *RACGAP1* (Rac GTPase activating protein 1) and *THBS1* (thrombospondin 1). All three genes had significantly upregulated sense transcripts and significantly downregulated antisense transcripts in SLE. The reversed change of sense and antisense transcripts of these genes suggested that the two transcripts are distinctly regulated.

LncRNA expression

LncRNAs can regulate nearby coding genes as *cis*-regulatory elements. We summarized the correlation of transcription between pairs of lncRNAs and coding genes (Figure S7). When the lncRNAs were located within 100 kb upstream of coding genes, the pairs had a positive correlation on average, regardless of whether the lncRNAs were on the same strand as the coding genes or not. The correlation increased as the distance between lncRNAs and coding genes became closer. On the other hand, when the lncRNAs were located downstream of coding genes, there was no association between the two classes of transcripts if they were on the opposite strands, and a much stronger association if they were on the same strand. The latter might suggest the misclassification of extended 3′ UTR transcription of coding genes as lncRNAs. Although lncRNAs were less likely to change in SLE compared to other RNA classes (Table S4), the locations of some significantly changed lncRNAs suggested their involvement in SLE. For example, both *HIVEP2* itself and a lncRNA about 800 to 1,500 bases upstream of its transcription start site (TSS) were significantly upregulated in SLE, although their RNA abundance was over 50 times different. There were also two significantly upregulated lncRNAs located within 50 kb of each other on chromosome 6 and surrounded by a group of signficantly dysregulated coding genes including *TAGAP*, *FNDC1*, *SOD2*, *WTAP*, and *ACAT2* (Figure S8).

Pri-miRNA expression

Our RNA purification method did not retain miRNAs, however, pri-miRNAs were easily detected and the combination of increased pri-miRNAs and decreased targets suggested an impact of the miRNAs (Figure S9). Two pri-miRNAs, miR 193a and miR-212, were statistically significantly increased in SLE samples with concomitantly diminished target mRNA levels. MiR-193a, regulating k-ras and survival, was increased by 776% ($p = 0.009$) [42]. MiR-212, regulating apoptosis, was increased by 148% ($p = 0.009$) [43]. Thesetwo cellular processes are known to be aberrant in SLE monocytes [44,45].

We validated mature miR-212-3p differential expression in SLE using qRT-PCR on new samples from six controls and 13 SLE patients (Figure S10). An exogenous control was used because we could not identify an miRNA in monocytes that was unaffected by cytokine treatment (data not shown). The expression of miR-212-5p was too low to be detected in this ligation-based detection strategy. The predicted targets of miR-193a-3p, miR-193a-5p and miR-212 were downregulated by 14.0% to 16.7% in SLE, which

was more than the average down-regulation of all other genes (Figure S11).

Repetitive element expression

Repetitive elements were globally downregulated in SLE (Figure 2C). A closer look at the downregulated repetitive elements showed that they were predominantly ERV classes and were closely correlated with each other, suggesting that they were co-regulated via an upstream mechanism. Interferon is known to repress retroviral activation and over-expression of interferon in SLE has been documented in several studies [1,2,46,47,48]. Coding genes having the highest positive correlation with the downregulated repetitive elements were potential targets of transcription factors AP1 ($p = 3.0E-8$), E47 ($p = 2.2E-9$), RFX1 ($p = 3.7E-8$), IRF1 ($p = 3.5E-2$), and IRF2 ($p = 4.3E-3$), also supporting an effect of interferons.

Isoform analysis

The proportions of the total reads assigned to each isoform for each gene were compared between the control and SLE samples to evaluate differential isoform transcription. Therefore, this analysis only considered the change of relative isoform abundance while ignoring the gene-level differential expression. This comparison identified 54 genes having one or more isoforms where the relative abundance was significantly changed ($p<0.05$) by at least 5%, such as *CCR2*, *HIVEP1*, *HIVEP2*, *IL1B*, *IL1R2* and *TLR2*. *SNAPC3* encodes a subunit of a protein complex that activates snRNA. It was previously known as having a single isoform, while our Cufflinks assembly identified a novel splicing site within its 3′UTR (Figure S12). The relative abundance of these two alternative transcripts was changed by about 12% with $p = 0.003$.

We observed that isoforms where the relative abundance was increased in SLE had significantly more exons than those with decreased relative abundance (Figure S13). This result indicates higher RNA splicing activity and/or more complex RNA processing in SLE monocytes.

We selected two biologically relevant genes to validate the expression of their novel isoforms in monocytes. Primers were designed to selectively bridge the novel exon-exon junction. *IL1R1* and *IRF8* were both found to include a large intragenic exon by RNA-seq (Figures S14 and S15). These exons have no protein coding potential. For IL1R1, the novel exon is an extra 5′ untranslated region. In both cases, PCR demonstrated incorporation of the novel exon (Figure S16).

Novel loci transcription

92% differentially expressed novel loci had higher transcription in SLE (Table S4). Many of the novel loci formed clusters located close to each other. For example, three of the four novel loci, located within a 5 kb region on chromosome 18, were among the most significantly upregulated transcripts (Figure S17). Another example was a cluster of 26 novel loci located within a 34 kb region on chromosome 8, most of which were significantly upregulated in SLE and none of which were downregulated (Figure S18). We selected 22 novel transcripts upregulated in SLE to be validated in new samples using qRT-PCR. Ten of the transcripts were significantly upregulated ($p = 0.034$ to 0.0006) in SLE (Figure 3A), and only two were not upregulated at all (Figure 3B). The other loci were upregulated without reaching statistical significance (Figure 3B and Figure S19).

The fact that these loci were preferentially or exclusively transcribed in SLE monocytes indicates they are very cell-specific. They were not present in seven annotated gene databases supporting SLE-specificity. To examine potential mechanisms driving expression, we initially treated MonoMac 6 cells with TNF-α, α2-interferon, γ–interferon, or LPS. Time course experiments identified the optimal stimulation time. Only LPS replicated the pattern of expression of novel loci seen in the SLE samples (Figure 4A). These findings were further validated using published datasets and examining concordance. In this analysis, genes over-expressed in SLE were equally likely to be represented in the a2-interferon- and LPS-induced genesets (Figure S20). We then confirmed the effect using monocytes from healthy adult donors (Figure 4B). To understand potential pathways regulating the LPS effect, we utilized p38 (SB203580), ERK (U0126) and JNK (SP600125) inhibitors. The major effect of LPS induction of the novel transcripts was p38 mediated (Figures 4C and 4D, Figures S21 and S22).

Circulating endotoxin

Although endotoxin has been previously implicated in murine lupus models and LPS is known to activate interferon pathways via TLR4, there has been no direct measurement of endotoxin in SLE patients [16,17,18,19,20,21]. We compared, therefore, serum levels from 99 female SLE patients and those from 112 female healthy adult blood donors. This patient set comprised new samples. The SLE patients were 91% female, 46% black, 54% Caucasian, mean age 37.1 years, mean SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) of 2.6 and mean physician global estimate of 0.45. The mean prednisone dose was 9 mg/day. The red cross blood donors were females who were self declared as healthy. SLE patients had significantly higher endotoxin levels compared to controls (Figure 5). When we examined clinical markers for associations with circulating endotoxin levels, we found no association with age, weight, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), specific organ damage, specific autoantibodies or complete blood count (CBC) parameters. Because LPS/endotoxin can induce type I interferon expression, we examined the concordance of coding genes expressed in SLE, after stimulation with LPS and after stimulation with α-interferon using our prior results and published array results [49,50]. There was substantial overlap, demonstrating that endotoxin can in part mimic the type I interferon signature seen in SLE (Figure S21).

Discussion

With the advent of next-generation sequencing technologies a more comprehensive and accurate transcriptional analysis has become feasible. We report a whole transcriptome analysis of patients with SLE and compare gene expression with that of healthy controls. We detected many instances of SLE-specific alternative splicing, alternative polyadenylation, and novel loci transcription. Splicing and polyadenylation in SLE both favored longer, more complex transcripts.

One of our goals was to identify the transcript abundance of non-coding RNAs, which have been demonstrated in subjects with Aicardi Goutières syndrome, an infantile-onset disorder with features of lupus, and in murine models, to drive a type I interferon signature [51,52,53]. In this study, we found instead decreased expression of many non-coding RNAs. We hypothesize that repression of endogenous retroviral sequences may be mediated by the type I interferon that is known to be overexpressed in SLE patients [54]. One class of non-coding RNA for which expression was clearly induced in SLE patients was that of the pri-miRNAs. These small non-coding RNAs are processed to repress translation and regulate multiple messenger

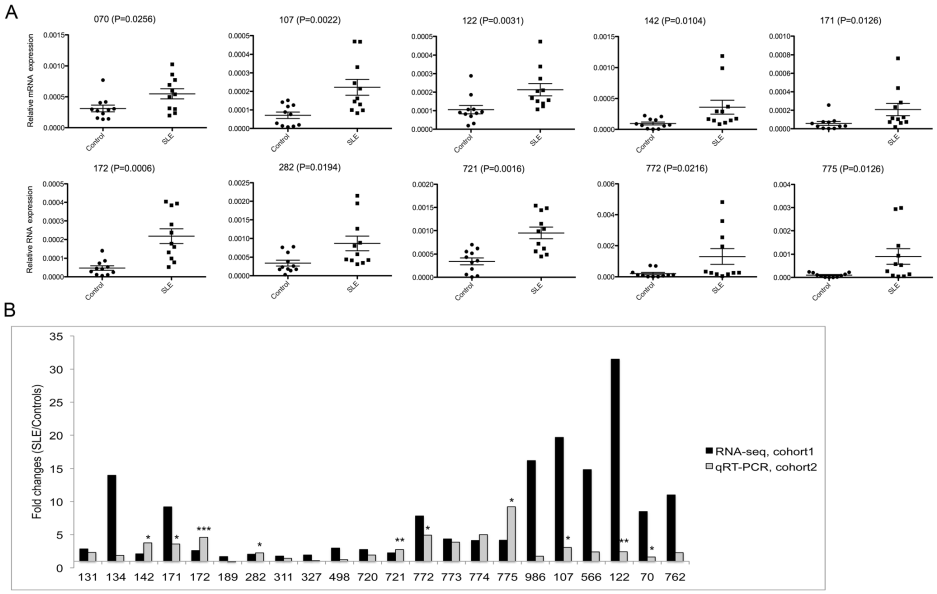

Figure 3. PCR validation in new samples. A) Ten novel loci (70, 107, 122, 142, 171, 172, 282. 721, 772, and 775) were amplified using 11 controls (8 new controls and 3 controls used for the RNA-seq libraries) and 11 new SLE patients. Transcript levels were normalized to β-actin. In each case, the differential expression between SLE and controls was statistically significant with $p<0.05$, according to the Mann-Whitney test. The cross bars indicate mean and standard error. Primers and locations are given in Methods S1. B) There was a good agreement of expression fold changes in SLE between the RNA-seq and qRT-PCR experiments with $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$(***). All but two of 22 tested novel loci were upregulated in both SLE patient cohorts.
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RNAs. In our study, two specific pri-miRNAs were significantly upregulated in SLE monocytes compared to healthy controls. In addition, we were able to demonstrate that the targets of these two miRNAs exhibited decreased message levels. These observations suggest that the elevated pri-miRNA levels that we identified in SLE monocytes are functionally relevant.

Among known protein-coding genes, there was evidence of global repression, and many genes downregulated in SLE monocytes were related to cell proliferation and cell adhesion. The genes upregulated in SLE monocytes reflected active inflammation. These observations are concordant with what has been seen using arrays and what is known about monocyte behavior in SLE [1,2,25,55,56,57,58,59]. Monocytes are known to have a shortened life span and to exhibit characteristics related to type I interferon exposure [1,23,45]. The overall repression of gene expression could be consistent with increased differentiation and our previous studies demonstrated altered surface expression of several proteins [14].

The finding of SLE-specific isoforms and polyadenylation was particularly intriguing. The finding of disease-specific isoforms is not unique to this study, but it has not been reported this extensively outside of tumor-specific transcripts. Some studies have found functional effects of autoantibodies directed at nuclear constituents and altered expression of splicing factors and this study raises the question of the mechanism driving altered processing [60,61,62]. Altered expression of splicing factors could also contribute to the pattern observed here [61,62].

This study raises many questions. We examined a cohort of patients with low disease activity to minimize the effect of medications. Whether patients with more severe disease could have a more disturbed transcriptome is not known. This is a relatively small cohort size, and additional studies will be required to replicate these findings. We did not perform extensive validation of the many classes of RNA found to have altered expression, instead capturing a snapshot of the SLE transcriptome in a single cell type and focusing on the breadth of the effect. We validated using new samples from controls and patients for the DEGs and found similar changes in nearly all DEGs, supporting that these effects are consistently seen in the disease. If similar disruptions are found in additional cell types, it would suggest a systematically altered transcriptome. Nevertheless, analyses of isoforms and unannotated loci is a moving target and additional information is likely to be forthcoming, allowing improved understanding of the regulation of these processes. Monocytes represent a cell type that is uniquely plastic and it may be that effects are magnified in this cell type.

IRF1, IRF2, and RFX1 were previously identified by us as potential regulators of genes with altered histone H4 acetylation [23,24,25]. These transcription factors, now identified as potential regulators of the SLE transcriptome, could integrate inflammatory and interferon signals. The MAP kinase and NFκB pathways were

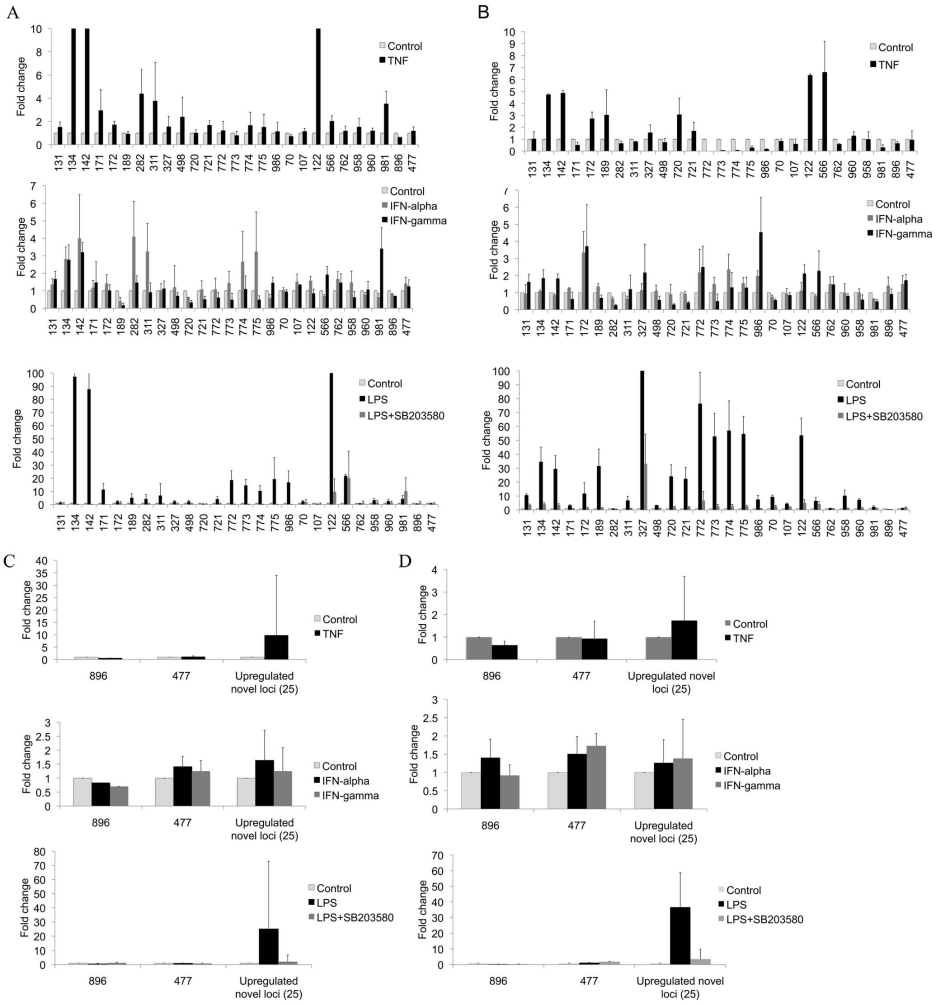

Figure 4. LPS stimulation of monocytes. A) MonoMac 6 cells were stimulated for 16 hours as indicated and qRT-PCR was performed for the novel loci. Only LPS (1 μg/ml) stimulation consistently upregulated these loci. TNF-α (10 ng/ml) led to upregulation of 16/25 (fold change>1.5), αIFN (100 U/ml) led to upregulation of 9/25 (fold change>1.5), γIFN (10 ng/ml) led to upregulation of 5/25 (fold change>1.5), and LPS led to upregulation of 22/25 (fold change>1.5). Three experiments with duplciates or triplicates were averaged. The error bars indicate standard deviation. B) Human primary monocytes were stimulated as above and the abundance of the novel transcripts quantitated by qRT-PCR. TNF-α led to upregulation of 9/25 (fold change>1.5), αIFN led to upregulation of 7/25 (fold change>1.5), γIFN led to upregulation of 9/25 (fold change>1.5), and LPS led to upregulation of 23/25 (fold change>1.5). Pre-treatment of cells with the p38 inhibitor, SB203580 led to markedly diminished induction of expression by LPS. Three experiments with duplciates or triplicates were averaged. The error bars indicate standard deviation. C) The average fold change for the aggregated novel upregulated loci in SLE were calculated for MonoMac 6 cells and D) healthy human primary monocytes stimulated by LPS, TNF-α, αIFN,or γIFN. Locus 896 was down-regulated and locus 477 was not changed in the orignal SLE samples and were included as controls. The error bars indicate standard deviation in C and D.
doi:10.1371/journal.pone.0093846.g004

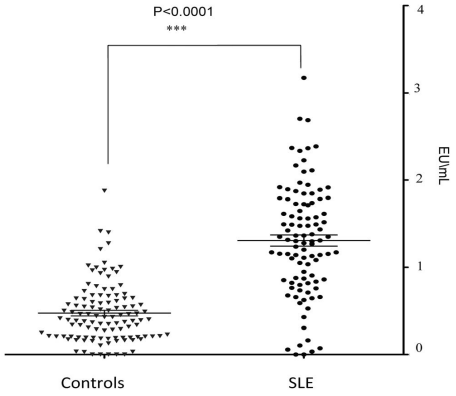

Figure 5. SLE patient circulating endotoxin levels. Circulating endotoxin was quantitated using the Limulus assay. 99 female SLE patients and 112 female Red Cross blood donors were anlayzed. SLE patients had significantly more endotoxin on average than controls ($P < 0.0001$). The cross bars indicate the mean and standard error.
doi:10.1371/journal.pone.0093846.g005

identified as potential regulating pathways in the polyadenylation pattern specific to the SLE samples. These pathways have long been implicated in SLE [63,64,65,66,67,68]. To further implicate the MAP kinase pathway, we found that expression of many novel loci were inducible with LPS and that inducibility could be blocked with the P38 inhibitor, SB203580. The roles of the novel loci are not yet known and we acknowledge that these fall into a bioinformatic limbo. Nevertheless, endotoxin has been implicated in a variety of diseases and induces type I IFN [69,70,71,72,73,74,75,76]. Our findings of significant overlap of the genes induced by LPS/endotoxin, SLE and α-interferon along with increased endotoxin in peripheral blood supports a role for endotoxin in the pathologic gene expression pattern identified here. This is the first RNA-seq study of SLE monocytes and our analysis revealed a surprisingly distorted transcriptome. The strengths of the study include the comprehensive approach to the characterization of the transcriptional landscape, use of a purified cell type, validation of the findings using a new cohort, and a mechanistic insight into the expression of novel loci.

Limitations of this study include a small sample size focused on mild to moderate disease activity. While the low disease activity enabled us to examine patient samples without the perturbation of high-level immune suppression, it may also have limited our findings. The platform used and the sample cell count may also have limited our findings. The libraries were 50 bp single reads and total RNA was used with post-run ribosomal RNA exclusion. Finally, technical aspects such as RNA quality may have limited our ability to identify disease-specific variation in signal. Nevertheless, in spite of these potential limitations, our analyses were robust and identified many changes specific to the SLE transcriptome.

In summary, we found a broadly altered transcriptome. By using a single cell type, we minimized the effects of different cell populations and improved the specificity of our discoveries. The most significant finding of this study was identification of disease-specific novel loci expression, regulated by endotoxin. Circulating endotoxin has been negatively associated with prognosis in a number of diseases and is thought to drive a type of immune exhaustion [77,78]. Whether endotoxin could be responsible for other features of the altered transcriptome or could represent a biomarker for disease severity remains to be determined. While additional studies will be required to determine which features contribute to the pathologic processes in this still enigmatic disease, the importance of this study lies in the identification of multiple features of altered transcription and processing in SLE, a heretofore unappreciated facet of the disease.

Supporting Information

Figure S1 Overall transcriptome characteristics. A) The Tophat-Cufflinks pipeline identified four major classes of transcripts from 17 RNA-seq libraries. RefSeq genes constituted the majority of the transcripts. B) Novel isoforms and loci were different from known transcripts in terms of average length and numbers of exons on average. C) Coding RNA was the most abundant RNA species (except ribosomal RNA) in monocytes based on the count of RNA-seq reads. Non-coding RNA collectively accounted for approximately 20% of total RNA. D) Small RNAs had the highest expression level on average after adjusting read counts for the total length of RNA classes.
(DOCX)

Figure S2 High novelty and high confidence novel isoforms and loci. A) Novel isoforms including at least one unknown exon-exon junction or mapped by ≥10 unique reads in at least six libraries were considering as having high-novelty or high-confidence, respectively. B) Novel loci not overlapping any known transcribed region or mapped by ≥10 unique reads in at least six libraries were considering as having high-novelty or high-confidence, respectively. Odds ratios and p values were the result of Fisher's Exact test performed on the overlap.
(PDF)

Figure S3 Validation of novel transcripts. Transcripts were validated for 27 identified novel loci using qRT-PCR (black bars). Primary monocyte RNA was used as the source and control amplifications using non-reverse-transcribed RNA were used as the negative control (No RT bars). Globin, not expected to be expressed in monocytes, was used as an additional negative control. Beta-actin was used for normalization. Most of these novel loci were generally expressed at low levels. The locations of the novel loci are in Methods S1. This represents n = 1. Further validation appears in the main text.
(DOCX)

Figure S4 Validation of differential gene expression. The differential expression of six coding genes in SLE were validated by qRT-PCR. The samples consisted of 11 controls (including 3 internal validation samples from which the RNA-seq libraries were made) and 11 new SLE patients. Five of the genes were validated as having significant change in SLE. The sixth gene, *CD177*, had the same direction of change in SLE samples but the change did not reach statistical significance. The cross bars indicate mean and standard error according to the Mann-Whitney test.
(DOCX)

Figure S5 3′ UTR length networks. A) The genes with longer 3′UTRs in SLE patients were networked using Ingenuity. The two most dominant networks are shown. NFκB, AKT, and UBC are the dominant nodes. B) The genes with shorter 3′UTRs in SLE patients were networked using Ingenuity. The two most dominant networks are shown. MAP kinases and UBC were the

dominant nodes. Data output from Ingenuity is shown in the Tables below.
(DOCX)

Figure S6 Sense- antisense expression. About 5,000 coding genes had detectable antisense transcription in monocytes. A) The average transcription of antisense transcripts was less than the half of the corresponding sense transcripts; but a small number of antisense transcripts had over ten-fold higher transcription of their sense counterparts. B) The transcription of sense and antisense pairs tended to be positively correlated. The correlation between each pair was first calculated across samples in the control and SLE groups separately, then the two correlation coefficients were combined using Fisher's transformation.
(DOCX)

Figure S7 LncRNA association with adjacent transcription. The co-regulation of lncRNAs and their nearby coding genes was dependent on their distance and relative location. The horizontal black line is indicates the average correlation of random pairs of lncRNAs and coding genes. (Oppo: opposite strand.)
(DOCX)

Figure S8 Chromosome 6 lncRNA cluster. A cluster of coding genes and lncRNAs located on chr6q25.3 were commonly dysregulated in SLE monocytes. Four coding genes with medium to high transcription levels were all upregulated in SLE while the other coding gene, *FNDC1*, had a very low transcription level and was downregulated in SLE. Both highlighted lncRNAs were significantly upregulated in SLE. Three other lncRNAs within this region had detectable transcription, but no significant changes in SLE. The Table demonstrates the read counts for each locus.
(DOCX)

Figure S9 MicroRNA- target correlation. Ten pri-miRNAs and their predicted targets had a negative correlation in monocytes. The correlation was calculated using normalized read counts of pri-miRNAs and target coding genes. The correlation coefficients of each miRNA-target pair were obtained from the control and SLE groups separately and then combined using Fisher's transformation. Each bar represents the average and standard error of correlation coefficients of a target list. Target lists were downloaded from miRBase.
(DOCX)

Figure S10 miRNA validation. The increased expression of miR-212-3p in SLE was validated by qRT-PCR with cel-miR-238 as a control. Six new controls and 13 new SLE samples were used.
(DOCX)

Figure S11 High expression miRNA target transcript expression. Three miRNAs whose pri-miRNAs had higher expression in SLE were examined for an effect on potential target transcripts. For these three miRNAs, target transcript expression was significantly downregulated on average. While coding genes were generally downregulated in SLE, the target genes tended to be downregulated even more. Target lists were downloaded from miRBase database.
(DOCX)

Figure S12 Isoform distribution for *SNAPC3*. A) Cufflinks assembly based on our RNA-seq data identified a novel splicing site within the 3′ UTR of *SNAPC3*. B) The relative abundance of the two isoforms was changed in SLE. C) The difference in relative abundance was statistically significant.
(DOCX)

Figure S13 Isoforms with increased expression in SLE are more complex. The isoforms where the relative abundance was increased in SLE had a higher number of exons than those where the relative abundance was decreased ($p = 0.007$). On average, the SLE-favored isoforms had about 0.5 exons more than the control-favored isoforms.
(DOCX)

Figure S14 Novel isoforms of IL1R1. Tophat-Cufflinks identified three isoforms of *IL1R1* not included in the RefSeq annotation. Two of them have been included in GENCODE database version 14 (**A&B**), and both had histone patterns at their transcription start sites consistent with expression according to ENCODE histone modification data sets generated from CD14+ monocytes (**C&D**). The other isoform, *TCONS_00030117*, had ~6 kb extra 5′ UTR exon (**E**). According to the ENCODE data, this exon has a strong H3K9me3 footprint (**F**), which is known as a repressive histone modification, suggesting unique transcriptional regulation at this region.
(DOCX)

Figure S15 Novel isoform of IRF8. Tophat-Cufflinks identified a novel isoform of *IRF8*, which included a prolonged exon 4. The existence of this isoform was supported by an ENCODE RNA-seq data set generated from 9 cell lines, including lymphoblastoid cell line GM12878. Transcription was detected in GM12878 cross the full gene body of IRF8, but the extended region of exon 4 in the novel isoform had higher transcription level than those of the introns. This isoform is likely an intermediate product of RNA processing and not present in the mature mRNA.
(DOCX)

Figure S16 Novel isoform validation. A) Tophat-Cufflinks identified multiple novel isoforms of *IL1R1*, each with a new exon-exon junction in the 5′ UTR. One of the isoforms (in red) was validated by qRT-PCR using a pair of primers across two exons (F4/R4). B) *IRF8* was known to have a single isoform. Tophat-Cufflinks identified a novel isoform, which was validated by qRT-PCR. These gels are representative of three experiments, with comparable results.
(DOCX)

Figure S17 Novel transcripts located on Chromosome 18. The detailed annotation of a cluster of novel transcripts located on chromosome 18 using public genomic data. A) Four novel transcripts identified by the Tophat-Cufflinks pipeline in monocytes about 20 kb upstream of coding gene *SERPINB2* are shown. All four transcripts were transcribed at the opposite direction as *SERPINB2*, and three of them were significantly upregulated in SLE by 303% to 671% ($p = 1.3E\text{-}6$ to $1.5E\text{-}8$). All four transcripts were validated by qPCR. B) These transcripts are not included in commonly used gene annotation databases. Two predicted genes partially overlap with these transcripts, but both are transcribed in the opposite direction. C) Sequences within this region are evolutionarily conserved. D) A very low level of transcription was detected within this region according to an ENCODE RNA-seq data set that measured transcriptomes in nine cell lines, not including monocytes. E) This region has a DNase I hypersensitive site in CD14+ monocytes according to another ENCODE data set. F) Furthermore, an ENCODE data set measuring various histone marks in CD14+ monocytes showed that this region has a nucleosome pattern indicative of active transcription.
(DOCX)

Figure S18 Novel transcripts located on Chromosome 8. A cluster of 26 novel transcripts located on chromosome 8 within a

~34 kb region upstream of coding gene *ADAM28*. A) All of the loci are transcribed in the opposite direction of *ADAM28*. B) None of them are included in a common gene annotation databases. Fifteen of these transcripts were significantly upregulated in SLE ($p<0.01$) by 80% to 3435% and none of them were downregulated in SLE. C) Most of the transcripts are located within evolutionarily conserved regions. D) ENCODE data detected very low levels of transcripts within this region in 9 cell lines. E) According to ENCODE data sets generated from monocytes, there is a DNase I hypersensitivity site within this region. F) The same sites showed a histone modification pattern of active nucleosomes.
(DOCX)

Figure S19 Differential expression of novel loci. Twelve novel loci were amplified using 11 controls (including three internal validation controls used for the RNA-seq libraries and eight new controls) and 11 new SLE samples using qRT-PCR. All genes were normalized to β-actin. P values according to Mann-Whitney in each case are given in parentheses. Only two failed to demonstrate increased expression in this new SLE cohort. Locations are given in Table S3. The cross bars indicate mean and standard error.
(DOCX)

Figure S20 Concordance of LPS, interferon and SLE gene expression. Five LPS data sets were created from four GEO monocyte data series (additional information on methods available in Methods S1). The α-interferon data set is from our previously published work. The coding genes shown to be upregulated in SLE were analyzed for upregulation after LPS stimulation and α-interferon (aIFN) treatment. A) All three LPS data sets demonstrated that LPS treatment also increased expression of the genes shown to be upregulated in SLE. N = the number of unique genes included in the analysis. B) The degree of overlap between SLE-induced genes and aIFN-induced genes was comparable to the degree of overlap between SLE-induced genes and LPS-induced genes.
(DOCX)

Figure S21 The effect of different MAP kinase inhibitors on LPS-induced novel loci expression. MonoMac6 cells (above) and primary monocytes (below) were treated with p38 (SB203580), ERK (U0126) and JNK (SP600125) inhibitors for 30 minutes and then stimulated with LPS. The y-axis represents the ratio of the LPS+ inhibitor-treated cells over the LPS alone treated cells, with the horizontal line indicating equivalence. The p38 inhibitor led to most diminished expression of the novel loci. At the right of the graph, loci 477 and 896 were included as controls, which were novel loci that were not altered in SLE. N = 3.
(DOCX)

Figure S22 The average of the novel loci expression. The expression levels of the novel loci, demonstrated in Figure S21, were averaged to better highlight the effect of the inhibitors. p38 (SB203580), ERK (U0126) and JNK (SP600125) inhibitors were used to define the role of this pathway on the novel loci expression. A) MonoMac6 cell results from Figure S21 were averaged to demonstrate the overall effect of the MAP kinase inhibitors. B) Similarly, in primary monocytes, the results from Figure S21 were averaged. TNF was used as a positive control for the LPS stimulation in C) MonoMac6 cells and D) primary monocytes.
(DOCX)

Table S1 Clinical characteristics of SLE patients.
(DOCX)

Table S2 RNA Samples for Libraries.
(DOCX)

Table S3 Locations of novel loci.
(DOCX)

Table S4 Number of differentially expressed genes/transcripts in each class.
(DOCX)

Table S5 Functional categorization of differentially expressed gene by DAVID.
(DOCX)

Table S6 Cross-referencing of genes identified in genetic association studies and significantly upregulated in this study.
(DOCX)

Data S1 Processed Data.
(XLS)

Methods S1 Supplemental Methods List.
(DOCX)

References S1 Supplemental References List: Tables.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KS. Performed the experiments: L. Shi KM L. Song. Analyzed the data: ZZ AY EA WW ZW PCR. Contributed reagents/materials/analysis tools: MP. Wrote the paper: KS.

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